PRE- AND POST-NATAL STRESS PROGRAMMING: FROM GENES TO PHYSIOLOGY

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General abstract

In a variety of vertebrate species, early life environmental cues are important drivers of an individual's phenotypic trajectories, priming physiological pathways, with consequences for growth, reproductive-related traits and lifespan. These phenotypic responses are believed to be adaptive in the short-term, but may impinge on health and survival over the long-term. Much of the work in this field has focused on the potential constraints imposed on animals after exposure to early life adversities, including nutritional deficit, sibling competition, and high predator pressure. Such stressful experiences can result in direct, but also indirect (via the maternal route) increases in the exposure to glucocorticoid stress hormones in the developing individuals. Glucocorticoids, whose production and secretion is regulated by the Hypothalamic-Pituitary-Adrenal axis (HPA axis), have been hypothesised to be the main candidates mediating the programming effects of developmental stress. Earlier predictions based on this assumption came from studies conducted in mammals. In mammals it is particularly difficult to manipulate exposure to circulating hormones in developing individuals because of the physiological intimacy between mother and offspring via the placenta and lactation. Here, I circumvent this complicating factor by using the precocial Japanese quail as a study species. In chapter 2 I measure corticosterone (B, the main avian glucocorticoid) stress responses to a standardised environmental stressor in growing quail aged 8- and 16-days-old. The results are consistent with those previously reported in other precocial birds, showing that the magnitude of the stress response (i.e. peak B within 30 min period) is higher in the 8- than the 16-day-old hatchlings. I find no differences in baseline B concentrations between the two groups. I then describe the main experiment in which I elevate B concentrations *in ovo* and/or in the endogenous circulation of the hatchlings (oral B administration from day 5 to day 19 post-hatching) in order to obtain four distinct phenotypes: pre-hatching B-treated birds, post-hatching B-treated birds, both pre- and post-hatching B-treated birds, and controls. I examine the specific and combined effects of pre- and post-hatching B on (1) growth trajectories and physiological stress responses before sexual maturity (post-hatch day 22) and upon adulthood (post-hatch day 64); (2) adult gene expression patterns within the hippocampus and hypothalamus, and (3) oxidative stress in the blood and the brain in the adults.

The main results of Chapter 3 show that post-hatching B, regardless of pre-hatching experiences, decrease HPA axis responsiveness in the juveniles, but only in the female quail; whilst pre-hatching stress, when not combined with post-hatching B, increase HPA responsiveness in both sexes upon adulthood. I also show that both pre- and post-hatching B induce short-term alterations in triglyceride basal concentrations, which are linked with the sex and basal glucose concentrations of the birds; the effects of pre-hatching B exposure were visible also upon adulthood with sex-specific alterations on basal glucose concentrations. Overall these results suggest that early life stress can trigger both transient and permanent physiological changes, depending on the sex and the quality of both the pre- and post-hatching environment. In Chapter 4 I show that the gene expression responses to pre- and post-hatching B are overall subtle, results similar to those reported in previous genomic studies that have manipulated early life rearing environments. The effects are, however, distinguishable, strongly tissue-specific and involve well characterised key candidate genes in the regulation of the HPA axis. These data also suggest important novel regulatory mechanisms, likely linked with cellular redox state, which may be driving the long-term effects of developmental stress. Finally, in chapter 5, I show that developmental B induces alterations in the basal antioxidant defences upon adulthood. The magnitude of these effects, once more, depends upon the timing of exposure, interactions between the pre- and post-hatching B and the tissue examined. As there are no differences in terminal oxidative damage, these results suggest that the B-treated birds could avoid oxidative stress via altering body oxidative defences. In summary, my findings throughout this thesis, illustrate the complexity of glucocorticoid programming and the importance of integrating analyses at multiple levels, from physiology to genome-wide investigations. The results of this thesis also strengthen the importance of examining the effects of early life stress over differing life stages in order to consider the overall balance of costs and benefits that may ultimately affect Darwinian fitness and survival.

Table of Contents

List of Tables

List of Figures

Patruus, Carlo, tibi.

Amor Natura, tecum.

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Author's Declaration

I hereby declare that the work presented in this thesis is entirely my own original work and carried out with the help of those people mentioned in the acknowledgements. The research work presented in this thesis has not been previously submitted to any other degree.

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1. Chapter

General Introduction

1.1 The concept of Stress

"Everybody knows what stress is and nobody knows what it is" is the perplexing comment posed by Hans Selye some forty years ago (Selye, 1974). The term "stress" is a notoriously broad concept in biology and the scientific community continues to make efforts to find an acceptable definition (Romero *et al.*, 2009).

The endocrinologist Hans Selye (1950) first coined the term "stress" and used this to include any condition that threatens an individual's homeostasis. Homeostasis encompass the capacity of living organisms to maintain a stable internal environment, including body temperature, blood glucose, pH levels, and water balance (Cannon, 1929). Selye also introduced the term "stressor" to indicate "the causative agents that trigger the stress response", defined as a cascade of emergency physiological and behavioural responses that re-establish homeostasis. Part of the problem with this definition derives from the inability to define rigorously the concepts of stressor, homeostasis and stress response. Recently, however, it has been widely accepted that any unpredictable event including environmental factors (e.g. extreme weather conditions, food restriction, and exposure to parasites) as well as behavioural factors, like social instability, can all be considered as stressors (Levine and Ursin, 1991).

Beyond the mere definitions, another problem in Selye's concept of stress is the lack of consideration of animal species life histories, hence, the dynamic phenotypic changes, involving growth, reproduction and lifespan, throughout the individuals' life cycle. The concept of "allostasis" (Sterling and Eyer, 1989; McEwen and Wingfield, 2003) is the first attempt to circumvent this weakness. Allostasis can be summarised as the process of maintaining homeostasis through changes in both environmental stimuli and physiological mechanisms and, therefore, takes into account the daily and seasonal physiological adjustments that constantly occur in the individual. However, it has been recently argued

that the allostasis model does not provide a framework to predict certain kinds of responses to stressful stimuli that may prepare ("prime" or "program") the individual to better cope with the future environmental conditions (Romero *et al.*, 2009). Examples of these potentially adaptive responses are those arising during development, when the individual is more especially sensitive to changes in anatomical structure and physiology (Seckl, 2001). It is widely accepted that early life experiences can potentially reset homeostatic settings and produce functional changes that can persist for the lifespan of the organism (reviewed by Seckl, 2004; Meaney *et al.*, 2007). To date, the Reactive scope model proposed by Romero and colleagues (2009) represents the first attempt to integrate within the notion of stress the importance of species' developmental strategies and their potential long-lasting effects in modifying future stress responses.

In summary, stress is a complex phenomenon and many mechanisms still remain to be addressed. It is intriguing to note that while it has been now over 60 years since Selye identified the main physiological mediator of coping with stressors, we still do not fully understand how the stress physiology helps animals to survive. This is a point of crucial importance for Darwinian selection because a significant part of the variation in fitness and longevity among individuals is likely to be linked to differences in their ability to deal with any perturbation to homeostasis.

1.2 The stress response and the biological relevance of the Hypothalamic-Pituitary Adrenal axis

All vertebrate taxa elicit a similar non-specific and rapid physiological stress response to cope with a variety of stressors (Cannon, 1929; recently reviewed by Chang and Hsua, 2004). Within seconds to hours after the perception of the stressor, two components of the stress response are activated, the first called the "fight or flight response", first described by Walter Cannon (1929), involving the immediate secretion of adrenalin from the adrenal cortex, and the second involving the activation of the Hypothalamic-Pituitary-Adrenal axis (HPA axis; Selye, 1974; Figure 1.1). The "fight or flight response" acts in the first seconds

and triggers a variety of physiological changes, including increased cardiovascular tone and respiration rate, that facilitate immediate physical reactions associated with a preparation for muscular action. Within minutes of the onset stressor, two neuropeptides from the paraventricular nucleus of the hypothalamus, corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP), act synergistically to stimulate the secretion of adrenocorticotropic hormone (ACTH) from corticotroph cells in the anterior pituitary gland. ACTH is then transported via the systemic circulation to the adrenal cortex, where it stimulates the production and secretion of glucocorticoid stress hormones (Nelson, 2005). In amphibians, reptiles and birds, the main biologically active glucocorticoid is corticosterone (B), whereas in most fish and mammals it is cortisol (among the exceptions are rodents, where it is B) (Harvey *et al.*, 1984). The resultant increase in circulating glucocorticoid concentrations initiates an array of metabolic changes that stimulate hepatic gluconeogenesis, inhibit glucose uptake by peripheral tissues and suppress inflammation and numerous immune reactions (Munck *et al.*, 1984). Such changes are thought to be adaptive, allowing the animals to move away from the source of danger and redirect physiology and behaviour towards immediate life-saving strategies, better known as "emergency life history stage" (Wingfield *et al.*, 1998). Avian research over the last decade has enormously contributed to elucidate the behavioural sub-stages mediating the development of the emergency life history stage and their links with stress hormones (Wingfield and Ramenofsky, 1997; Wingfield *et al.*, 1998). In detail, the first event involves the deactivation of territorial behaviour and disruption of social hierarchies (Wingfield and Silverin, 1986; Wingfield *et al.*, 1998; Meddle *et al.*, 2002), which could impinge on reproduction. For example, implants of B in free-living pied flycatchers (*Ficedula hypoleuca*) reduced parental care or resulted in complete abandonment of nests depending on the hormonal doses implanted (Silverin, 1986). The second sub-stage is the activation of the emergency behaviour, which allows the animal to activate the appropriate behavioural strategy to respond to the perturbation, such as seek a refuge to hide, or leave and find an alternate habitat depending on the encountered environmental conditions (e.g. Astheimer *et al.*, 1992; Breuner *et al.*, 1998b). The last sub-stage is characterised by the termination of the emergency life history stage and recovery phase that allow the animal to return to its normal life history state (Astheimer *et al.*, 1992). Importantly, such

sub-stages are not avian-specific but rather widespread across vertebrate taxa (recently reviewed by Wingfield and Romero, 2010).

An adaptive stress response usually involves relatively low initial glucocorticoid concentrations that reach a physiological peak within minutes (usually 10-15min) of exposure to a stressor, with glucocorticoids returning to the previous baseline after the exposure to a stressor terminates (reviewed by Cockrem, 2013). The short-term nature of the stress response is, therefore, very important (e.g. Wingfield *et al.*, 1998; Sapolsky, 2000). Glucocorticoids play a major role in switching off the stress response. Indeed, the positive top-down regulation of adrenocortical activity is counteracted via bottom-up negative feedback actions of glucocorticoids that bind to specific intracellular receptors in various neuronal structures, especially in the hippocampus and hypothalamus (Bons *et al.*, 1976; Bradbury *et al.*, 1994; de Kloet *et al.*, 1996). There are two types of corticosteroid receptors: the higher affinity mineralocorticoid or type I (herein referred as MR) receptor and the lower affinity glucocorticoid or type II (herein referred as GR) receptor (Reul and de Kloet, 1985). Such different binding affinities of glucocorticoids to their receptors are thought to play a key role in the dynamic modulation of the stress response (Oitzl *et al.*, 2010). Prolonged or repeated stimulation of the HPA axis, for instance via repeated exposure to stressful stimuli, can compromise the efficiency of negative feedback on the HPA axis, leading to chronically elevated circulating stress hormones. Elevated glucocorticoid concentrations over longer periods can be damaging and cause inhibition of the reproductive axis, suppression of the immune system, impairment of growth, increased cellular oxidative stress and neuronal cell death (Sapolsky, 1992; McEwen and Stellar, 1993; de Kloet *et al.*, 2005a; Costantini *et al.*, 2011a). However, there is also a growing proposal among behavioural endocrinologists and evolutionary ecologists that a prolonged elevation of stress hormones might be advantageous during specific life stages (e.g. long period of starvation during migration, persistent exposure to high predation pressure), and therefore, favoured by natural selection. Clearly, such advantages will depend on the overall balance between fitness benefits (e.g. increasing the likelihood of reproduction) and costs (e.g. increased risk factors for disease) throughout an animal's lifespan (Monaghan, 2008).

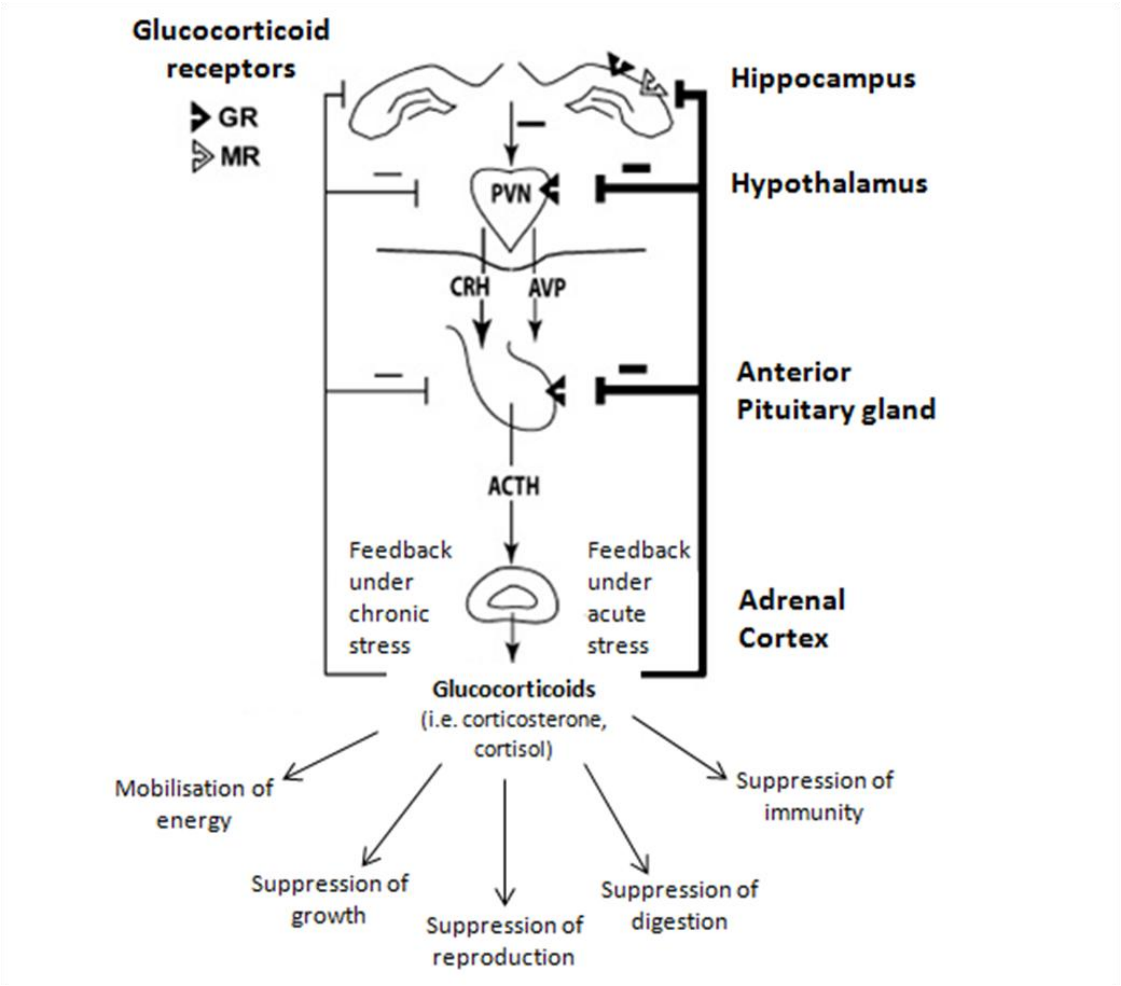

Figure 1.1. Regulation of the Hypothalamic-Pituitary-Adrenal axis (HPA axis) in the mammalian brain. Briefly, after the perception of a stressor, the hypothalamic paraventricular nucleus (PVN) releases corticotrophin releasing hormone (CRH) and vasopressin (AVP), which stimulate the release of adrenocorticotrophin hormone (ACTH) in the anterior pituitary. ACTH in turn stimulates the production and secretion of glucocorticoids (corticosterone or cortisol depending on the species) from the adrenal cortex. Elevated glucocorticoids exert an array of metabolic and behavioural effects in order to maintain body homeostasis. The HPA axis is tightly regulated over time via negative feedback loops (indicated by the sign -) on mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) in the brain and anterior pituitary. Under acute stress conditions, feedback mechanisms operate efficiently and the effects of elevated glucocorticoids are only short-term (within hours). Under chronic stressful conditions, feedback mechanisms are impaired causing prolonged activation of the HPA axis, with potential detrimental consequences on body processes. In the brain, MR have a higher affinity than GR for glucocorticoids. Therefore, at basal concentrations of glucocorticoids, MR are occupied whereas GR remain largely unoccupied. During acute stress, there is increased occupation of GR. Hippocampal MR are thought to be primarily involved in feedback regulation during basal secretion, while GR become important during stressful conditions. From de Kloet *et al.*, 1999; Matthews, 2002; Sapolsky, 2002 (see also paragraph 1.2 for more detailed information on the HPA axis). Figure from Broonstra, 2004.

1.2.2 The importance of the HPA axis for the study of the long-term effects of stress on the phenotype

In contrast to adrenalin, glucocorticoid hormones are lipophilic; which means that they can readily cross the blood-brain barrier and directly bind to corticosteroid receptors (Reul and de Kloet, 1985). Since the endocrine mediation of the stress response must affect the brain in order to affect behaviour, glucocorticoids are believed to be ideal candidates to study the long-term effects of stressful conditions on the individual's phenotype. Given the lack of consensus on the biological definition of stress (Section 1.1), it is important to clarify that, in this study, stress refers the HPA axis system that is activated in response to exposure to a stressor, and a stress response only occurs in response to an increase in glucocorticoid synthesis and secretion into the blood.

1.3 The role of the early environment

Early life experiences are key drivers of phenotypic trajectories and life history strategies (Mousseau and Fox, 1998; Monaghan, 2008). In early life, environmental factors can act directly, or indirectly, on the phenotype. Indirect effects are commonly known as "maternal effects" and refer to the ability of the mother, to influence the phenotypic development of her offspring (Mousseau and Fox, 1998; Wolf *et al.*, 1998). Hormones are thought to be the main candidate mediators of such direct and indirect environmental effects on the phenotype. The first evidence supporting this idea was put forward by Charles H. Phoenix's seminal study demonstrating that female guinea pigs exposed to exogenous testosterone as embryos, gonadectomised before puberty and treated with testosterone as adults exhibited masculinised sexual behaviours (i.e. mounting responses) (Phoenix *et al.*, 1959). This study, coupled with later research carried out by Young and collaborators (e.g. Young *et al.*, 1964), have posed the basis for the so termed "*organisational-activation hypothesis*". This hypothesis highlights the importance of (1) the environmental stimuli during critical developmental periods, and (2) the dual action of testosterone in "organising" tissues and neuroendocrine pathways in early life *vs.* "activating" appropriate sex-specific responses later in life. A series of follow-up studies have

progressively expanded Phoenix's hypothesis more broadly at different levels of biological organisation (cellular, molecular and genetic) and confirmed that the organisational and activational effects of sex steroid hormones can explain variations in a large variety of phenotypic traits, also involving non-gonadal tissues (reviewed by Arnold, 2009). Furthermore, there are now evidence showing that in vertebrates sexually dimorphic traits are due to a combination of gonadal hormones and the direct effects of genes encoded on the sex chromosomes (reviewed by Arnold, 2004). For example, a notable experiment by Gahr (2003) in the Japanese quail (*Coturnix c. Japonica*) demonstrated that when genetically female hypothalamic tissue was transplanted into the body of a male, testicular growth and testosterone secretion were lower than in genetic males receiving transplants of genetically male hypothalamus. These results suggest that the genetic sex of brain cells has important organisational effects, which can constrain the cell's functional phenotype and the normal development of the hypothalamic-pituitary-gonadal axis.

1.3.1 Early life stress and the concept of glucocorticoid programming

Numerous experiments between 1970 and 1980 have focused attention on the potential organisational-activational role of stressful environmental stimuli during development on the phenotype. A key study in this field showed that male offspring of rats born from stressed mothers (i.e. using combination of restraint and light) exhibited demasculinisation and feminisation of sexual behaviours, with decreased ejaculatory pattern and increased lordotic behaviour (Ward, 1972). Similar effects were confirmed later by another laboratory group (Dahlöf *et al.*, 1977, 1978). Remarkably, it was later demonstrated that Ward's restraint protocol imposed on the pregnant rat induced elevated B levels in both the mother and its offspring (Ward and Weisz, 1984). Such results can be interpreted as the first experimental evidence demonstrating that stressed mothers can potentially produce stressed offspring phenotypes.

Following Ward's earlier findings, several biomedical researchers and epidemiologists have focused on the long-term phenotypic effects associated

with stressors experienced during both the pre- and post-natal development. For example, a large body of literature in humans has suggested consistent correlations among low birth weight, infant feeding or adverse socio-economic conditions during infancy with an increased propensity to a wide range of metabolic disorders into adulthood, notably hypertension, insulin resistance, type 2 diabetes and cardiovascular diseases (e.g. Barker *et al.*, 1990, 1993; Lissau and Sorensen, 1994; Lithell *et al.*, 1996; Forsdahl, 1977; recently reviewed by Godfrey *et al.*, 2007). In light of the original Phoenix's hypothesis and Ward's findings, glucocorticoids and changes in the HPA activity have been hypothesised to be the main drivers of such effects via programming mechanisms occurring during development that persist throughout life. This phenomenon is today more generically known as *pre-natal glucocorticoid programming or peri-natal glucocorticoid programming*" (Seckl, 2001, 2004; Meaney *et al.*, 2007; Cottrell and Seckl, 2009).

1.3.1.1 Experimental studies on pre-natal/pre-hatching stress

Accumulating evidence across a variety of vertebrate taxa, from fish to mammals, show that stressed mothers with elevated endogenous glucocorticoids can expose their embryos to these circulating stress hormones through the placenta or their presence in the egg (reviewed in Henriksen *et al.*, 2011). For example, in birds, Hayward and Wingfield (2004) were the first to provide experimental evidence in an avian model (the Japanese quail), that adult females implanted with B produced eggs with higher concentrations of yolk B than control females. The concentration increase was observed after 1 week the treatment started, which is a time compatible with the time interval required for yolk formation in pre-ovulatory follicles (Hayward and Wingfield, 2004). Similar findings were found later in the barn swallow (*Hirundo rustica*), with females exposed to predators laying eggs with higher B concentrations in the albumin compared to control females (Saino *et al.*, 2005). In the latter study the increase was observed the day after the start of the treatment (Saino *et al.*, 2005), which is the expected time range of steroid hormones deposition in the albumen post-ovulation (Warren and Scott, 1935). Although the literature on glucocorticoid-mediated maternal effects is growing in birds, to date the

majority of the studies have mainly focused on maternal androgens (reviewed by Groothuis *et al.*, 2005; Groothuis and Schwabl, 2008). Such studies have contributed to demonstrate that elevated yolk androgens can lead to both short-term effects on offspring behaviour, such as enhanced nestling growth and begging rates (e.g. Schwabl, 1996; Eising *et al.*, 2001; Eising and Groothuis, 2003; but see Sockman and Schwabl, 2000; Pilz *et al.*, 2004) and long-term effects in adult exploratory behaviour as well as the development and expression of sexually dimorphic traits (Ruuskanen and Laaksonon, 2010; Schweitzer *et al.*, 2013). Despite the consolidated knowledge in birds that maternal steroid hormones accumulate in the egg, there are still limited information regarding the exact mechanisms underlying their transfer and deposition to the eggs. In contrast to sex steroids that are produced locally in the cell layers of the follicular wall surrounding the growing oocyte during vitellogenesis (Bahr *et al.*, 1983; Okuliarova *et al.*, 2010), glucocorticoids need to be transported from the adrenal glands to the oocyte via the blood circulation, and how exactly this occurs remains to be answered.

Beyond the mechanisms of transfer of maternal stress hormones to the offspring, there are a line of experimental evidence, from fish to mammals, showing that pre-natal/pre-hatching elevated glucocorticoids can shape a wide array of phenotypic responses, including alterations in growth trajectories, cognition and competitive abilities, stress-related behaviours both in wild and captive animals' populations (e.g. fish: Sloman, 2010; reptiles: De Fraipont *et al.*, 2000; birds: Hayward and Wingfield, 2004; Rubolini *et al.*, 2005; mammals: review by Weinstock, 2008). To date, only a surprising small number of studies, and mainly in birds, have examined the effects of elevated maternal pre-natal stress on the activity of the offspring HPA axis. The results from these experiments showed that elevation of maternal plasma B concentrations or direct elevation of B *in ovo*, as well as a range of stressful protocols can all have the potential to induce long-term alterations in the dynamics of the offspring stress responses post-hatching (e.g. Hayward and Wingfield, 2004; Hayward *et al.*, 2006; Love and Williams, 2008; Haussmann *et al.*, 2012). To the best of my knowledge, there is only one study in birds that examined the effects of pre-hatching stressful manipulations (mimicked via *in ovo* injection with B) on corticosteroid receptors (MR and GR), and exclusively on GR (Ahmed *et al.*, *in press*). The latter study

found a diminished expression of the GR protein content in the hypothalamus of pre-hatching B-exposed chickens when adults in comparison with the controls, but no differences were found in GR gene expression within the same brain area (Ahmed *et al.*, *in press*). However, these effects were observed only when a high dose of B was injected in the egg yolk, whereas no effects were seen in the birds that were exposed to a low dose of B pre-hatching (Ahmed *et al.*, *in press*). Although these results are promising, more studies across different vertebrate species looking at both GR and MR receptors in the brain will be needed to clarify the potential long-lasting changes of these systems in response to pre-hatching stressful conditions.

The effects of pre-natal stress on the HPA system have been studied in much more details in laboratory rats. These studies lead to the common assumption that maternal pre-natal stress produce offspring that, as adults, exhibit increased HPA axis responsiveness, with steeper increases in peak glucocorticoid concentrations and a slower return to baseline levels in response to acute stress (reviewed by Kapoor *et al.*, 2006). These alterations in the stress response have been associated with weaker negative feedback capacity due to a decreased number of GR and MR receptors in the hippocampus of the adult offspring (Barbazanges *et al.*, 1996; Levitt *et al.*, 1996; Welberg *et al.*, 2001; Emack *et al.*, 2008). Recent reviews of the available mammalian literature, however, highlighted many inconsistent patterns across studies, probably due to different intensity and timing of the stressor, sex and the age of the offspring (e.g. Weinstock, 2008; Henriksen *et al.*, 2011). For example, studies in rats and mice indicate that long-term alterations in the programming of the HPA axis of the offspring appear only if the maternal stressor occurs at least once daily between days 14 and 21 of gestation (e.g. Henry *et al.*, 1994; Maccari *et al.*, 2003; Koenig *et al.*, 2005), suggesting the presence of specific sensitive windows in which the elevation of glucocorticoids can induce long-lasting changes in the developmental trajectories (reviewed by Weinstock, 2008). Similar pre-natal critical periods appear to exist also in guinea pigs (Kapoor *et al.*, 2006) and common marmoset (Pryce *et al.*, 2011). Such pre-natal sensitive windows are thought to be caused by differences in the ability of the embryo HPA axis to respond to maternal stressors during gestation and are thought to be especially dependent on the appearance of MR and GR in the brain (Weinstock, 2008).

However, this hypothesis to date remains to be experimentally tested. Another factor that could contribute to explain the high variations across the mammalian literature regards the potential mechanisms that can regulate/counteract the effects of maternal pre-natal glucocorticoids on the offspring (Shams, 1998; Seckl and Meaney, 2004). In fact in mammals, it is now well established that the access of maternal glucocorticoids to the embryo is partially regulated by the type 2 isoform of the placental enzyme 11ß-hydroxysteroid dehydrogenase (11ß-HSD2). This isoform catalyses the rapid inactivation of active glucocorticoids (B or cortisol) into their inert metabolites (11-dehydrocorticosterone or cortisone respectively) (Murphy *et al.*, 1974). Placental 11ß-HSD2 activity has been shown to vary significantly among individuals in both rats (Benediktsson *et al.*, 1993) and humans (Stewart *et al.*, 1995). Experimental work in rats demonstrated that acute stress on pre-natal day 20 up-regulated placental 11ß-HSD2 activity by 160% (Welberg *et al.*, 2005). The latter study also showed that maternal chronic stress experienced during the third week of pregnancy (pre-natal days 14-19) did not alter placental 11ß-HSD2 activity, but it reduced the capacity to up-regulate the enzyme in the placenta by 90% when the dams were faced with an acute stressor (Welberg *et al.*, 2005). Moreover, a study in mice showed that 11ß-HSD2$^{-/-}$ offspring of either 11ß-HSD2$^{+/-}$ or 11ß-HSD2$^{-/-}$ mothers had reduced birth weight and exhibited higher anxiety than 11ß-HSD2$^{+/+}$ littermates, providing evidence for the key role of feto-placental 11ß-HSD2 in pre-natal glucocorticoid programming (Holmes *et al.*, 2006). Altogether these data indicate that 11ß-HSD2 is likely to play a key role in modulating glucocorticoid access from the mother body to the embryo, a mechanism potentially evolved to protect the embryo from some of the negative effects observed in pre-natally stressed individuals. This idea appears reinforced by recent studies that have found 11ß-HSD2 also in the ovary of zebra finch (*Taeniopygia guttata*) (Katz *et al.*, 2010) and in the gonads and oviduct of chickens (*Gallus gallus*) (Klusonova *et al.*, 2008), suggesting that the presence of this enzyme could have the same function as in mammals also in egg-laying vertebrate species. Overall these data also upgrade the role of the embryo in the response mechanism underlying pre-natal programming: the general idea of the embryo as a passive receiver with the mother having the windward in the parent-offspring conflict appears now outdated (Carere and Balthazart, 2007; Groothuis and Schwabl, 2008). Therefore, in conclusion, despite the large amount of studies in mammals, many

questions, such as developmental windows, interactions between variation in maternal HPA axis activity and different embryonic responsiveness to the effects of glucocorticoids, and especially, the proximate mechanisms underlying the long-lasting effects on offspring physiology and behaviour, remained mainly unresolved.

1.3.1.2 Experimental studies on post-natal/post-hatching stress

After birth/post-hatching, several stressor types (i.e. sibling competition, low food provisioning, maternal separation or direct glucocorticoid administration) can lead to increases in endogenous glucocorticoids in the growing offspring across a variety of vertebrate groups (e.g. reptiles: Meylan *et al.*, 2002; birds: Kitaysky *et al.*, 1999; Spencer *et al.* 2003; Spencer *et al.*, 2009; mammals: Rosenfeld *et al.*, 1992). These consequent alterations in endogenous stress hormone concentrations have been shown to produce changes in several phenotypic traits, such as song, competitive and dispersal behaviour (e.g. Meylan *et al.*, 2002; Spencer *et al.*, 2003; Spencer and Verhulst, 2007); and, especially, alterations in the adult HPA responsiveness and/or in the gene expression of corticosteroid receptors in a brain-region specific fashion (Spencer *et al.*, 2009; Banerjee *et al.*, 2012). In fact, Banerjee and collaborators (2012) showed that adult zebra finches that hatched from maternally-deprived nests had diminished GR mRNA expression levels within the hypothalamus when compared to the non-maternally deprived adults, but no treatment differences were observed within both the hippocampus and cerebellum on GR; on contrast, MR mRNA levels in the maternally-deprived birds were lower than in the control group within all these brain regions. Again, as for the studies in birds on pre-hatching stress (Paragraph 1.3.1.1), the latter work is, to the best of my knowledge, the only experimental work that looked at the long-term effects of post-hatching stressors (although not using a direct manipulation with B) on the corticosteroid receptors in the brain and more studies are needed to elucidate the proximate mechanisms.

The abundant literature of post-natal stress programming in rats primarily involves alterations in mother-pup interactions. For instance, adult rat offspring

born from mothers who naturally exhibit high levels of care were found to show dampened HPA stress responses, as well as elevated expression of GR receptors in the hippocampus and decreased expression of CRH in the paraventricular nucleus of the hypothalamus (Liu *et al.*, 1997). These adult offspring were also less fearful in comparison to the adults that as juveniles experienced low levels of maternal care. Importantly, such observed phenotypic changes could be reversed by cross-fostering the biological offspring of high- and low-care mothers (Francis *et al.*, 1999). On the other hand, prolonged separation of the pups from the mother causes the opposite effects on the adult offspring HPA axis, with enhanced stress responsiveness (Plotsky and Meaney, 1993), underscoring the importance of considering the type and intensity of the stressor when considering the potential effects on the adult phenotype.

At least in mammals and similarly as occurs during the pre-natal development (see Paragraph 1.3.1.1 above), the effects of glucocorticoid hormone exposure during post-natal development on the nervous system and behaviour appear to be stronger during specific post-natal sensitive periods. Although most of the research on this field focused on the effects of steroid hormone exposure during pre-natal or early post-natal development, it has been suggested that adolescence (broadly defined as the period of life that includes attainment of sexual maturity; Spear, 2000) can also be another critical sensitive period of life (Spear, 2000; recently reviewed by Brown and Spencer, 2013). However, studies focused on the effects of adolescent stress on HPA axis development show variable outcomes (reviewed by McCormick *et al.*, 2010), and again, such large variation in the results may be explained by differences in stressor types and intensity. Although all vertebrates undergo the critical transition from an immature state (pre-puberty) to one that is capable of reproduction, only a few studies in species different from mammalian models investigated the effects of stress specifically during puberty/adolescence. This is partially due to the difficulties in establishing a clear/discrete separation in the continuum of the physiological processes occurring between pre-puberty and puberty in several vertebrate species, especially in seasonally breeding species with intermittent reproductive activation (reviewed by Perfito and Bentley, 2009). The few studies carried out in birds suggest that stressful manipulations specifically during adolescence, such as housing conditions or unpredictable light:dirk cycles, can

have the potential to modulate both stress reactivity and behaviour in later life (Heiblum *et al.*, 2000; Lindqvist *et al.*, 2007). Although these studies highlight the importance of this period of life also in bird species, more studies are needed to examine whether the underlying effects of the observed changes in the latter studies are determined directly by glucocorticoids and not by indirect changes (and interacting effects) in other hormones, such as sex steroids (reviewed by Brown and Spencer, 2013).

1.3.1.3 Interaction between pre-natal/pre-hatching and post-natal/post-hatching stressors

Overall the literature suggests that pre- and post-natal/hatching environments can impinge on the same behavioural and endocrine pathways associated with the HPA axis system. Lines of evidence suggests that environmental experiences occurring during these two developmental periods can have different long-lasting effects on the adult phenotype. More importantly, the effects of pre-natal stress can also depend on the quality of the post-natal rearing environments. For example in rats, post-natal handling can reverse the increase in emotional reactivity or the effects induced by pre-natal stress (Wakshlak and Weinstock, 1990). A similar suppression of the effects of pre-natal stress in the rat model has been reported on the HPA axis activity as a consequence of post-natal adoption (Maccari *et al.*, 1995). These experimental data have emphasised the importance of considering interactive stimuli occurring throughout the differing stages of development when investigating the effects of developmental stress. At the same time, they reinforce the hypothesis that the HPA axis may be the main biological substrate for such interactions (Maccari *et al.*, 1995).

In order to test this hypothesis experimentally, however, it would be important to control and, hence, to be able to manipulate direct and indirect environmental components acting on the growing individuals. As it has been recently pointed out, in mammals the relatively inaccessibility of the embryo, as well as the prolonged physiological intimacy between the mother and offspring via the placenta and the transfer of milk challenge direct experimental

manipulations on the developing individuals (Spencer *et al.*, 2009; Henriksen *et al.*, 2011).

1.3.1.4 The advantages of egg-laying vertebrates and the bird as a model

Egg-laying vertebrates have been shown to be better models for conducting direct manipulations of the pre- and/or post-natal environment (fish: McCormick, 2000; reptiles: Meylan *et al.*, 2002; birds: Love and Williams, 2008). In fact, the egg once deposited/laid represents a sealed environment and it is content may be manipulated with none or very minimal direct influences of the mother, with the exception of her incubation behaviour (Groothuis *et al.*, 2005). This physical separation between the mother and the embryo also facilitates descriptive research to investigate, for example, correlations of nutrients and hormones deposited in the egg with the mother's body conditions during egg formation. Also, post hatching, the lack of direct maternal hormone transfer between the mother and its offspring through lactation allows better controlled experimental manipulations of endogenous glucocorticoids in the juveniles (e.g. Spencer *et al.*, 2009). As discussed earlier (Paragraph 1.3.1.1), birds are a classical model to study hormone-mediated mechanisms of maternal effects (recently reviewed by Groothuis *et al.*, 2005; Groothuis and Schwabl, 2008). First, there are substantial evidence showing that steroid hormones, including glucocorticoids, are deposited into the yolk of the egg over the course of 1 week before laying (e.g. Hackl *et al.*, 2003; Hayward and Wingfield, 2004; Almasi *et al.*, 2012) and approximately over the last 24h into the albumen (e.g. Warren and Scott, 1935; Conrad and Scott, 1938; Hackl *et al.*, 2003; Saino *et al.*, 2005).Therefore, the hormone yolk content is considered a good proxy of the physiological state of the mother over a relatively long timeframe; whereas the albumen is limited to a much shorter range period. Importantly, both yolk androgen and glucocorticoid concentrations respond to artificial selection for behaviour (Gil and Faure, 2007; Hayward *et al.*, 2005), suggesting that hormone deposition in the egg may be a trait under natural selection. Second, the neuroendocrine and endocrine regulatory pathways, including those related to the stress physiology and HPA axis, have been well characterised in several bird species and showed high degree of similarities with mammals (Wingfield, 2005a). Several tracing and functional experiments in different bird species have

demonstrated connectivity throughout steroid-sensitive brain areas, such as those within the hypothalamus, midbrain and amygdala (e.g. Briganti *et al.*, 1996; Balthazart and Absil, 1997; Cheng *et al.*, 1999; Riters and Alger, 2004), and a variety of steroid-dependent behaviours, including parental behaviour, aggression and sexual responses (e.g. Balthazart *et al.*, 1998; Thompson *et al.*, 1998; Ruscio and Adkins-Regan, 2004). Such connectivity shows high degree of similarity with mammalian findings (reviewed by Goodson *et al.*, 2005). Importantly, investigations on immediate early gene responses in birds (using antibodies for Fos and Zenk, also known as egr-1) have shown that birds and mammals exhibit similar patterns of activation following agonistic encounters (Goodson and Evans, 2004) and copulatory behaviour (Ball *et al.,* 2007; Charlier *et al.,* 2005). Such parallel between neuroendocrinology and behaviour in mammals and birds facilitates comparative research in a more adaptive and evolutionary framework (Henriksen *et al.*, 2011).

1.4 The study species: the Japanese quail

The Japanese quail (*Coturnix c. japonica*) belongs to the order of Galliformes and the family Phasianidae and has been domesticated since the 11th century in China from wild populations in Eastern Asia (Huss *et al.*, 2008). The Japanese quail is an ideal model for the study of developmental stress. First of all, recent studies carried out in Japanese quail have demonstrated that the yolk B content of the egg can be successfully manipulated via injection of known physiological doses of exogenous B soon after laying, without significant effects on embryonic mortality (Hayward *et al.*, 2006; Boogert *et al.,* 2013). Second, fertile eggs are artificially incubated and this eliminates sources of variations, for instance those due to parental incubation efforts and assures an ideal standardisation of experimental condition throughout the pre-hatching stages of development. Third, the precocial development of quail prevents post-hatching maternal hormonal input. In fact, the juveniles are able to walk, see, hear and feed themselves independently soon upon hatching. In natural conditions, post-hatch maternal care are characterised by brooding behaviour during approximately 2 weeks of age (Mills *et al.*, 1997). In captivity, brooding temperature can be successfully simulated using heat lamps for the first 2 weeks of post-hatching

life. Using the Japanese quail, therefore, I was able to manipulate experimentally the quality of the post-hatching environment via directly administrating exogenous doses of B within the relevant physiological ranges in the absence of the potential confounding factor of maternal care. Furthermore, puberty in this species is reached relatively rapidly, between 6-8 weeks of age, with females maturing slightly later than males (Ottinger, 2001). Overall the easiness to breed quail and reliably in captivity as well as their rapid development has made this species a widely used model in laboratories all over the world and a great deal of the endocrine and neuroendocrine systems, including those relating the stress physiology, have been already described and confirmed high similarities with other vertebrate groups, especially mammals (Ottinger *et al.*, 2001; 2004). Finally, quail are often a preferred model in behavioural endocrinology than the domestic chicken due to their smaller size, but also because quail have not been intensively selected by poultry industry for specific traits, such as egg or meat production as the chicken (Ball and Balthazart, 2010). The recent sequencing of the chicken genome, a species closely related to quail, is of great importance to, at least in part, overcome the limitation due to the relatively limited genetic information in quail.

1.5 Outline of the thesis

The overall aim of this thesis was to examine how and the extent to which, developmental stress influences individuals' phenotypic trajectories and HPA axis programming in later life using the Japanese quail as study species. The use of this avian model allowed me to experimentally alter the quality of both the pre- and post-hatching environment mimicking exposure to standardised stressors and analyse the potential short- and long-term interactions between pre- and post-hatching exposure to B on the HPA axis phenotype. This research combines traditional methods to measure hormonal and cellular stress responses coupled with the most recent high-throughput technologies to measure global gene expression patterns in target brain tissues. The use of this integrative approach has strengthened my ability to collate a wide range of scientific approaches together that allowed me to further the understanding of overall regulatory patterns underlying stress. More specifically, the objectives of this thesis were to address the following questions:

- Can Japanese juvenile quail mount a stress response? If yes, does this ability change with age during post-natal development (Chapter 2)?

- Does pre- and post-hatching exposure to B cause changes in growth patterns, HPA responsiveness, glucose and triglyceride stress responses in juvenile and adult quail? Do the short- and long-term effects of developmental stress differ (Chapter 3)?

- Does pre- and post-hatching exposure to B cause long-lasting gene expression pattern changes in the hippocampus and hypothalamus into adulthood (Chapter 4)?

- Does pre- and post-hatching exposure to B cause long-lasting changes in the body oxidative balance in the adult quail (chapter 5)?

I finally conclude the thesis with a general discussion in which the results of these experimental studies are related to previous findings and potential future directions are also identified (Chapter 6).

2. Chapter

Post-hatch age-related changes in the stress response in developing Japanese quail

2.1. Abstract

Vertebrate species respond to stressful environmental conditions by activating a stress response under the control of the Hypothalamic-Pituitary-Adrenal axis (HPA axis). During an individual's development, the HPA axis undergoes phases of maturation, which are likely to be co-evolved with the specific developmental strategy ("*Developmental hypothesis*"). The large variation of life histories in birds within the altricial-precocial range makes this taxon an excellent model to study such interactions in a comparative framework. Here, I examine the HPA axis responsiveness in Japanese quail hatchlings aged 8- or 16-days by measuring baseline and stress-induced corticosterone (B) concentrations during a standardised environmental stressor. I also estimate the strength of correlation between B and the birds' morphometric traits (body mass, structural size and body condition) in both the age classes. I find that the magnitude of the stress response is higher in the 8-day-old hatchlings than in the 16-day-old birds. There are no differences in baseline levels between the two age groups. The results also suggest links between stress-induced B levels with body mass and body size in the younger hatchlings, but not in the older birds. These patterns support the few studies conducted in other precocial species and fit well within the *"Developmental Hypothesis"*. The age-related decline in the stress responsiveness in precocial birds may be the optimal trade-off to respond promptly to environmental stressors during the earlier stages of development and to minimise excessive loss of energy later on life when the birds may be less vulnerable to stressors, such as predator attacks.

2.2 Introduction

Living organisms respond and adapt to the current environmental conditions by adjusting their physiology, behaviour or morphology (reviewed recently by Wingfield, 2013). In vertebrate species, the ways in which such individuals' responses are regulated depend upon a variety of factors, such as the genetic background, sex, body condition, and social interactions (Wingfield, 2008). An individual's age may also be a critical factor in the modulation of such responses, especially during the early life stages (Monaghan, 2008). In fact, the capacity of growing animals to perceive environmental signals and, consequently, transduce these cues into appropriate neural and hormonal responses can be influenced by internal factors, for example the individual's ontogeny, or by external factors determined by the physical environment.

A physiological system that is likely to play a key role in transmitting environmental signals is the Hypothalamic-Pituitary-Adrenal axis (HPA axis; Sapolsky, 1992). The HPA axis regulates the release of glucocorticoid stress hormones from the adrenal glands into the blood circulation. Temporary rises in glucocorticoid concentrations result in the mobilisation of body energy resources (i.e. glucose and triglycerides) that enhance immediate survival, suspend ongoing activities and allow individuals to move away from the perturbation, such as a predator attack or inclement weather (Breuner and Hahn, 2003; Breuner *et al.*, 1998b; Wingfield *et al.*, 1998; Sapolsky *et al.*, 2000). Protracted activation of the HPA axis, however, can lead to chronically elevated circulating concentrations of glucocorticoids, with potential detrimental effects on the immune system, reproductive activities and brain functioning (Sapolsky, 2000; de Kloet *et al.*, 2005a). The individual, therefore, needs to be able to efficiently modulate the release of baseline and stress-induced stress hormones, trading-off the need for mounting the stress response and the necessity to limit excessive and unnecessary loss of energy. Importantly, in the majority of vertebrate species (i.e. reptiles, amphibians, birds, and mammals; Romero, 2002), such re-allocation of energetic resources follows distinct seasonal patterns. For example, in several passerines baseline and stress-induced corticosterone (B) concentrations (respectively in 72% and 86% of the species), the primary active glucocorticoid in birds, is highest during the breeding season and lowest during

the pre-basic moult (reviewed by Romero, 2002; see also Liebl *et al.*, 2013). An up-regulated adrenocortical responsiveness may be an adaptive strategy for breeding animals to be more responsive towards predator attacks and assure protection to their offspring (i.e. *"Energy Mobilisation Hypothesis"* - Ketterson and Nolan, 1999; Romero, 2002); while, on the other hand, a down-regulated HPA axis during moulting may help individuals to maximise investment in feather quality, probably by suppressing the protein catabolic activity of glucocorticoids (Romero *et al.,* 2005). Such seasonal fluctuations in the adrenocortical sensitivity and reactivity, however, are likely to differ or be more complex depending on the specific life history of the species. For example, several Arctic breeding birds show attenuated, or even suppressed, B stress responses during the breeding season (e.g. Wingfield *et al.*, 1994a, b; Silverin *et al.*, 1997; Silverin and Wingfield, 1998) and have been shown to be insensitive to the behavioural effects of high B levels (Astheimer *et al.*, 2000; Meddle *et al.*, 2001; Meddle *et al.*, 2003; although see Meddle *et al.*, 2002). Moreover, the breeding stage and the sex can also play an important role in the modulation of adrenocortical responsiveness. For example, the Arctic breeding male Smith's longspurs (*Calcarius pictus*) do not show any attenuation of their HPA axis early in the breeding season on arrival at their breeding grounds during territorial establishment, but do show a diminished stress response later during the parental phase, when, intriguingly, moult also occurs (Meddle *et al.*, 2003).

Despite the large body of literature, especially in birds, on the physiological variation underlying physiological stress responses, most of this work has been carried out in adult individuals (recently reviewed by Cockrem, 2013). The effects of stressful conditions during pre- and/or post-natal development can have permanent effects on developmental processes, including impaired growth and long-term alterations in growth trajectories (e.g. Morici *et al.,* 1997; Spencer and Verhulst, 2007; Spencer *et al.,* 2009), sexual differentiation (e.g. Ward and Stehm, 1991), adult stress responsiveness (e.g. Hayward and Wingfield, 2004; Spencer *et al.,* 2009; Banerjee *et al.,* 2012; Marasco *et al.,* 2012 or Chapter 3 in this thesis) as well as changes in stress-related adult behaviours (Maccari *et al.,* 1995; Davis *et al.,* 2008). Newborn altricial laboratory rodents display a hypo-responsive HPA axis during their first two weeks of post-natal life, with low and stable B concentrations and a diminished

stress response (Sapolsky and Meaney, 1986; Levine, 1994). A similar HPA axis quiescence has been reported in altricial and semi-altricial birds during the early post-natal developmental windows (e.g. Romero *et al.,* 1998; Sims and Holberton, 2000; Love *et al.,* 2003; Wada *et al.,* 2007; reviewed by Wada, 2008). In contrast, in precocial birds, the HPA axis appears to be functional much earlier in life, at least from the pre-hatching mid-incubation stages (Tanabe *et al.,* 1986). Moreover, while non-precocial young birds often show maximal or similar adult-like stress responses as fledglings (Love *et al.,* 2003; Walker *et al.,* 2005; Wada *et al.,* 2008), precocial hatchlings exhibit elevated baselines and maximal HPA responsiveness at hatching (Kalliecharan and Hall, 1976; Tanabe *et al.,* 1986; Carsia *et al.,* 1987), which tend to decline with increasing post-natal age (Wentworth and Hussein, 1985; Carsia *et al.,* 1987; Holmes *et al.,* 1989; Dickens and Romero, 2010). Overall, such inter-species variation patterns suggests that the physiological capacity of a juvenile bird to deal with the environmental challenges depends upon the degree of post-hatching parental dependence as well as its capacity to thermoregulate, locomote and forage independently (i.e. *"Developmental Hypothesis"*- Schwabl, 1999; Kitaysky *et al.,* 2003; Blas *et al.,* 2006; Wada, 2008) . Therefore, altricial birds that hatch almost naked, blind and are fully dependent on their parents are predicted to exhibit little or no glucocorticoid release in response to the environmental perturbations experienced as nestlings; while precocial birds that can feed independently from their parents fairly soon after hatching are predicted to develop the HPA axis earlier than altricial species in order to appropriately respond to the external environment.

The aim of the current study was to examine the post-natal development of the adrenocortical responses to a standardised environmental stressor in the precocial Japanese quail. Specifically, the main objectives were (*i*) to examine if quail hatchlings were able to mount a B stress response to a standardised environmental stressor; if yes, (*ii*) to test whether such capacity would differ across differing stages of post-natal development, and finally, (*iii*) to investigate the potential relationships between the stress response, morphometric traits and energetic conditions in the growing individuals. These objectives were accomplished by evaluating the ability of the quail hatchlings to release B in response to a standardised capture-restraint-stress protocol (Wingfield *et al.,*

1982) at post-natal day (PN) 8 and PN16, during the linear phase of growth, and analysing the strength of potential correlations between body mass, structural size and body condition with both baseline and stress-induced B. I predicted that the adrenocortical activity during a standardised restraint stress protocol would decline with post-natal age due to the highly precocial nature of Japanese quail kept in captive conditions (i.e. *"Developmental hypothesis",* see above for references). Also, I expected that B (both baseline and stress-induced levels) would negatively correlate with the morphometric traits measured since glucocorticoids are known to promote energy expenditure and can impair increases in body mass and structural size (Sapolsky *et al.*, 2000). Due to the limited literature in juvenile bird species and the high variation in developmental mode in this taxon, it is hard to predict whether baseline B levels would be a more relevant biological parameter than stress-induce B levels to explain variations in morphometric traits. However, a recent comparative study in breeding free-living males of a large variety of passerine species suggested that stress-induced B levels may be a stronger predictor of body mass than baseline B levels (Hau *et al.*, 2010).

2.3 Material and Methods

The animal work was conducted at the Cochno Farm and Research Centre, University of Glasgow, UK. All indoor rooms were climate controlled at 19°C. All the eggs used for this experiment were derived from the in-house breeding stock. Breeding quail (n = 10 females and 5 males) lived in trios (2 females:1 male) in 79 X 48 X 58cm enclosures. For the present experiment, a total of 45 eggs were collected, marked according to maternal identity (identified by colour and marking patterns) and incubated (incubator Ova-Easy 190A, Brinsea Products, Sandford, UK) at 37.5^{o}C and 55% humidity while being turned twice hourly. Of 45 eggs, 33 eggs were fertile (73%) and 25 quail hatched (76%). At hatching, quail were labelled with unique colour combinations using nail varnish applied on the feathers and placed back in the incubator to allow the plumage of the birds to dry. All the experimental quail were sexed *post-mortem* as this cannot be achieved by plumage pattern before 2-3 weeks of age (see below). Between 24 and 36h after hatching all the juveniles were housed in brooders,

each divided into smaller compartments using cardboard dividers. Quail housed in the same brooder compartment (n = 2 or 3) were age-matched and randomly assigned to one of the following experimental groups: (a) PN8: standardised capture-restraint-stress protocol at post-hatching day 8 (n=13, females: 8; males: 5); (b) PN16: standardised capture-restraint-stress protocol at post-hatching day 16 (n = 12; females: 10; males: 2). PN8 and PN16 were chosen because these two time points are within the period in which the juveniles show a steep phase of linear growth (e.g. Chapter 3, Figure 3.1) and modulation of adrenocortical responsiveness during this time is likely to be biologically relevant. Furthermore, PN8 was the earliest time point in which I could obtain enough plasma from each individual bird for the hormonal analyses. The birds were tested at specific ages to minimise possible confounds due to HPA axis activity changes, which are known to occur, especially during development (e.g. Schwabl, 1999). Brooding temperature was 35.5^{o}C with a daily decline from PN3 of 1-1.5^{o}C until the end of the experiment. Nail varnish markers were replaced with individual leg-bands in the hatchlings allocated to the PN16 experimental groups when they reached 7-8 days. Food (turkey starter crumbs, Dodson and Horrell, Kettering, Northamptonshire, UK) and water were provided *ad libitum*; all the animals were kept on a 12h:12h light:dark cycle (lights on 7am-7pm).

2.3.1 Standardised capture-restraint-stress protocol

The day prior to the standardised capture-restraint-stress protocol, body mass for each bird was measured to the nearest 0.1g using a balance (Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, UK); tarsus and head plus bill lengths were measured to the nearest 0.1mm using a digital calliper (Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, UK). The following day, the hatchlings were removed from their brooders between 8.30 and 12.49h and a blood sample (approximately 75µl) was immediately collected by brachial venipuncture into heparinised capillary tubes within approximately 1.5min (= T0, mean ± s.e.m., 1.20min ± 0.11) of capture. Since B titers in birds do not start to rise until 2-3min after capture (Romero and Reed, 2005), all these initial blood samples were considered to reflect baseline concentrations. Importantly, all the birds (n = 2 or 3) housed within the same

brooder compartment were caught and bled at the same time; birds housed in different brooders were visually isolated in order to assure minimal disturbance during the capture procedure. Each bird was then placed into a cardboard box (15 X 15 X 12cm), which was placed over a mini brooder (Brinsea Products, Sandford, UK) to keep the hatchlings warm and two further blood samples were taken approximately at 10min (= T10, mean ± s.e.m., 10.72min ± 0.15) and 30min (= T30, mean ± s.e.m., 30.60min ± 0.14) following capture after which time the birds were returned to their brooder. In the majority of bird species, plasma B concentrations peaks at 10-15min after handling and declines at 30-40min (Cockrem, 2013; Wall, 2010 in the Japanese quail). Therefore the measurements taken at T10 and T30 were expected to represent the magnitude of the stress response and the recovery efficiency to baseline, respectively. Blood samples were kept on ice for up to 4h before being centrifuged and plasma withdrawn and stored at 20^{o}C for later hormonal analyses. The experiment ended the following day when the birds were sacrificed by cervical dislocation or by intra-peritoneal administration of 1ml of Euthatal (sodium pentobarbital, 200mg/ml; Merial Animal Health, Harlow, UK).

2.3.2 B Radioimmunoassay

Assays for measuring plasma B concentrations were conducted at the School of Veterinary Medicine, Jarrett Building, University of Glasgow, UK. All the B assays conducted for the work of this thesis employed a double antibody Radioimmunoassay along with at least 1 standard curve of known amounts of B (concentration range: 20ng/ml–0.038ng/ml), following a similar protocol to that described in Wingfield *et al*. (1991). Specifically, B was first extracted from 20µl of plasma using 10mm diameter glass tubes (Fisher Scientific Ltd, Loughborough, Leicestershire, UK). In order to measure the recovery efficiency (estimate of the accuracy of the extraction procedure), tracer amounts (≈3000 cpm) of [1, 2, 6, 7–3H] B label (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK) were added to each sample and incubated for 1h at 4ºC. Then 1ml of diethyl ether (Rathburn Chemicals, Walkerburn, UK) was added to each tube and samples were placed on dry ice for 5min. The obtained supernatant from each sample, containing the extracted B, was decanted into an empty glass tube. Samples

were then allowed to dry at 40ºC using a sample concentrator (Techne, Scientific Laboratory Supplies, East Riding of Yorkshire, UK) and air block (KFN Neuberger, Oxfordshire, UK). Once dried, samples were reconstituted with 300µl of assay buffer (0.01M PBS, 0.25% BSA; pH = 7.4) and maintained at 4ºC for at least 2h before proceeding with the next assay steps. Then, 50µl of each extracted sample was placed into plastic assay tubes (size 3.5ml, Sarstedt, Leicester, UK) with 1ml scintillation liquid (National Diagnostics, Atlanta, Georgia, USA), and counted in a liquid scintillation analyser (Packard, 1600 TR) to measure the recovery efficiencies. Subsequently, four plastic assay tubes for each of the following were set up: (1) "Totals" containing 100µl of B label (≈10000cpm) in 200µl assay buffer; (2) "Non-specific binding" containing 100µl of B label in 200µl assay buffer; (3) "Maximum binding" containing 100µl of B label, 100µl of primary antibody (anti-B antiserum, code B3-163, Esoterix, Austin TX - dilution 1: 100 in assay buffer and Normal Rabbit Serum, Sigma-Aldrich, Dorset, UK - dilution 1:400 in assay buffer). Tubes containing either samples (100µl, in duplicate) or standards (100µl, in triplicate) also received 100µl of primary antibody (same solution as with the maximum binding tubes), followed by 100µl of B label. In each assay, together with the quail samples, chicken plasma and two-B-spiked chicken plasma pools that gave approximately 80, 70 and 50% binding on the standard curve, respectively, were included as quality controls. The tubes were then vortexed and incubated at 4ºC for 24h. The following day, 100µl of a second antibody (Goat anti-rabbit IgG, Sigma-Aldrich, Dorset, UK), diluted 1:50 in assay buffer, was added to all the tubes except to the totals. The tubes were then vortexed before being incubated at 4ºC for a further 24h. Following this, 400µl of microcellulose (Sigmacell Cellulose, Type 20, Sigma-Aldrich, Dorset, UK) diluted in assay buffer (0.1g/100ml) was added to all the tubes except to the Totals. The tubes were then spun for 50min at 2000rpm and the supernatant aspirated. The remaining pellets were reconstituted with 50µl of 0.1M sodium hydroxide, vortexed and 1ml of scintillation fluid was added before counting on the counter. Counts obtained for the standards and unknown samples were analysed and converted to concentrations in the unknown samples using the universal assay calculator Assay Zap (version 2.69, Biosoft, Cambridge, UK).

2.3.2.1 B analyses

Extraction efficiencies estimated for each sample averaged (mean ± s.e.m.) 84.77% ± 0.01. Samples collected at each age were run in two separate assays and the mean assay sensitivity was 0.2ng/ml. The intra-assay coefficients of variation were 25% and 4%, while the inter-assay coefficients of variation at 80, 70 and 50% binding were 8%, 17% and 17%, respectively and were calculated using the chicken quality controls (as explained in Paragraph 2.3.2). Comparability between the two assays was assured by the quality controls, which were within the expected range of concentrations in both assays, as well as by interpolating the standard curves performed in each assay.

2.3.3 Statistical analysis

Data analysis was performed in PASW statistics 19.0 (Armonk, NY: IBM Corp.) using Linear Mixed Effect models (LMEs) fitted by restricted maximum likelihood. One female in the PN8 group was excluded from the analysis because of the lack of a T10 sample. Potential age-differences in the dynamics of the stress response (i.e. T0, T10 and T30) were investigated using a repeated-measure approach with age, time of sampling and their interaction as fixed factors, while mother identity was included as an additional random factor to control for pseudo-replication due to the presence of hatchlings sharing the same mother. Age-related differences in the adrenocortical activity were further examined in three separate LMEs using the following response variables: (a) baseline B (i.e. T0 samples); (b) peak B (i.e. difference between the highest B concentrations at either T10 or T30 minus baseline B concentrations) and (c) the change in B between T10 and T30. Peak B and the change in B between T10 and T30 are considered good estimate of the magnitude of the stress response and the recovery to baseline levels, respectively. In all the LMEs, age was included as fixed factor and mother as random factor. To meet the assumptions of the LME modelling, B concentrations measured across the stress response as well as peak B were $\log_{10}$-transformed for normality; all model residuals were normally distributed. Sex was never included as a factor in the analyses because sample size was female-biased, especially in the PN16 group. However, the same model

performed in the dataset after excluding the data from the males from both PN8 and PN16 groups showed the same significant statistical outcome. Moreover, in preliminary analyses potential sex-differences in the adrenocortical responses within the PN8 group of birds were examined by performing a LME with sex, time of sampling and their interaction as fixed factor, while mother was included as a random factor; neither sex nor its interaction with time of sampling were significant in the model ($p > 0.3$ for both).

An index of body size for each individual bird was estimated by extracting the first component scores from a Principal Component Analysis (PCA) with tarsus length and head plus bill length (PCA: eigenvalue = 1.90; total variance = 95.06%). Body condition (i.e. body mass corrected for body size) was then calculated as the residuals from a linear regression between body mass and body size, similarly to previous studies (e.g. Love *et al.*, 2005; Angelier *et al.*, 2009). The relationships between (1) body mass, (2) body size, (3) tarsus length, (4) head plus bill length and (5) body condition with B were then tested using Pearson's tests, which were carried out separately by age and by sampling time. Similarly as before, these correlations were performed without splitting the data by sex. Sexual dimorphism in body mass values in our quail population started to appear after the third week of post-hatching life (see Table 3.2 in Chapter 3). In fact, in the PN8 group, there were no differences in any of the body measurements recorded between the two sexes (*t-test*: $0.08 < p < 0.67$). In the PN16 group, the statistical outcome did not change when removing the 2 males from the dataset. However, as the data in the PN16 group are female-biased I am unable to exclude potential sex-related differences between B and the morphological data recorded.

2.4 Results

2.4.1 HPA axis responsiveness at PN8 and PN16

B concentrations during the capture-restraint protocol were significantly affected by age and by sampling interval (age: $F_{1,\ 21.12} = 12.48$, $p = 0.002$; sampling interval: $F_{2,\ 35.89} = 71.07$, $p = 0.0001$, Figure 2.1). There were no

differences in baseline B concentrations between the two age classes ($F_{1,\ 21.96}$ = 0.564, p = 0.461). However, the dynamics of the stress response across the 30-min period differed between the two age classes (interaction age X time of sampling: $F_{2,\ 35.89}$ = 4.44, p = 0.019). Specifically, as illustrated in Figure 2.1, in the 8-day-old hatchlings B concentrations increased and peaked at T10 and on average remained stably high until T30, whereas in the 16-day-old hatchlings B concentrations peaked at T10 and declined at T30. Age was a significant predictor of the magnitude of the stress response ($F_{1,\ 21.57}$ = 6.38, p = 0.019), with 8-day-old hatchlings showing significantly higher peak B concentrations than 16-day-old hatchlings (Figure 2.2). There were no differences between the two age classes in the change in the hormone concentration between T10 and T30 ($F_{1,\ 20.13}$ = 1.22, p = 0.283).

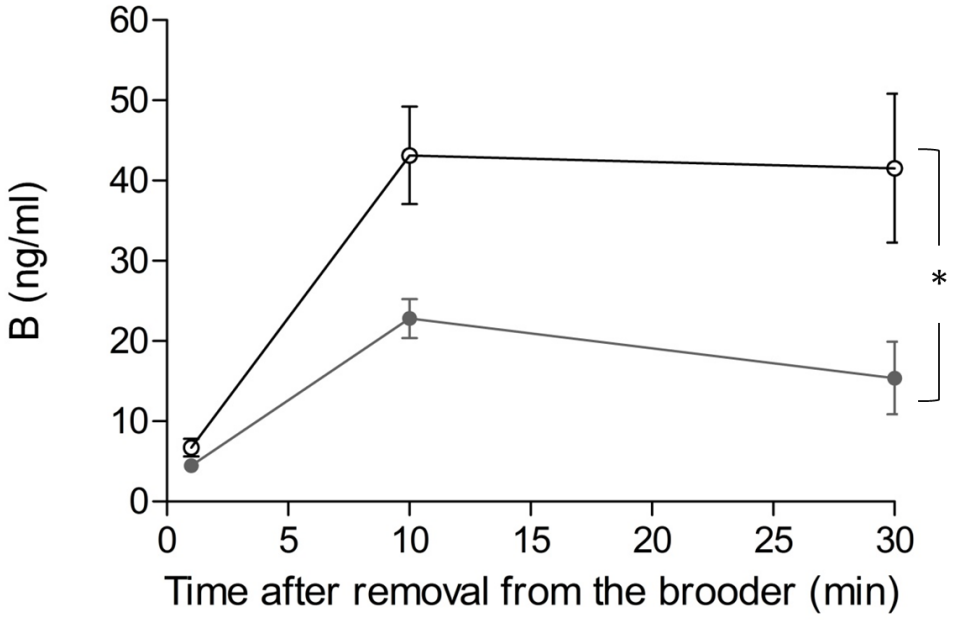

Figure 2.1. Changes in corticosterone concentrations (B) in Japanese quail aged 8 (open circles) or 16 days (filled circles) during a standardised restraint-stress 30 min protocol. Data are shown as un-transformed means ± s.e.m. Linear Mixed Model: age x time of sampling interaction, p = 0.02; * indicates significant differences. Sample sizes: PN8 = 12, females: 7, males: 5; PN16, females: 10, males: 2.

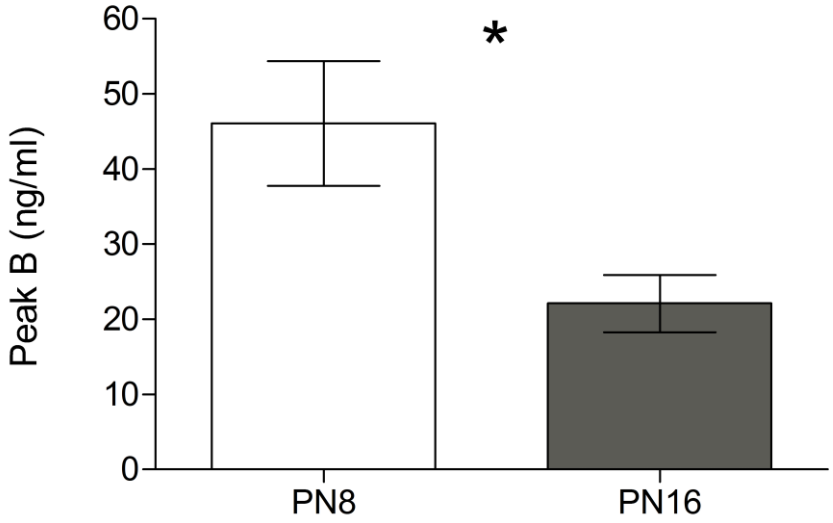

Figure 2.2. Difference between the highest corticosterone (B) concentrations (either T10 or T30) and baseline (peak B ng/ml) in Japanese quail at post-hatching day (PN) 8 and PN16 during a standardised restraint stress 30 min protocol. Linear Mixed Model: age, p = 0.02; * indicates significant differences. Data are presented as un-transformed means ± s.e.m.

2.4.2 Correlation of morphometry and body condition with B at PN8 and PN16

In the PN8 experimental group there was a significant negative correlation between stress-induced concentrations of B at T10 and body mass (r_s = -0.601, p = 0.039, Figure 2.3), but there were no significant correlations of these two parameters at T0 and T30 (full statistics reported in Table 2.1). Similar significant relationships were found between B concentrations at T10 and tarsus length and body size (Figure 2.3, tarsus: r_s = -0.696, p = 0.012; body size: r_s = -0.765, p = 0.004; in Table 2.1 for full statistics on the other parameters). Head plus bill length was not correlated with the variation in the stress hormone levels over the stress response in the 8-day-old juveniles (Table 2.1, Figure 2.3); despite this, the overall body size strongly co-varied with stressed-induced B concentrations (T10: r_s = -0.765, p = 0.004, Figure 2.3).

In the 16-day-old juveniles there were some significant relationships between B concentrations and specific morphological traits at T30 (Table 2.1). However, as can be seen from the figures (Figure 2.4), the strength of these correlations was strongly biased by 2 highly influential observations from two individual females

(0.84 < Cook's distance < 2; Cook, 1977; Bollen and Jackman, 1990) and the significance disappeared when they were removed from the dataset.

Table 2.1 Correlations (Pearson's tests) between corticosterone (B) baselines (i.e. T0) and stress-induced B concentrations (i.e. T10 and T30) across a standardised restraint-stress 30 min protocol and morphometric measures (i.e. body mass, tarsus length, head plus bill, body size), and body condition in Japanese quail hatchlings aged 8- and 16 days (PN8 or PN16, respectively). Outcome in bold indicate significant correlations ($p < 0.05$).

PN8			**PN16**		
Body mass	r_s	*p*	Body mass	r_s	*p*
T0	-0.320	0.310	T0	-0.230	0.472
T10	**-0.601**	**0.039**	T10	-0.143	0.658
T30	-0.493	0.103	**T30 ***	**-0.666**	**0.018**
Tarsus length	r_s	*p*	Tarsus length	r_s	*p*
T0	-0.468	0.125	T0	-0.370	0.237
T10	**-0.696**	**0.012**	T10	0.018	0.955
T30	-0.528	0.077	**T30 ***	**-0.668**	**0.018**
Head plus bill length	r_s	*p*	Head plus bill length	r_s	*p*
T0	-0.076	0.815	T0	-0.039	0.904
T10	-0.494	0.102	T10	-0.134	0.678
T30	-0.077	0.812	T30	-0.320	0.311
Body size	r_s	*p*	Body size	r_s	*p*
T0	-0.308	0.330	T0	-0.180	0.575
T10	**-0.765**	**0.004**	T10	-0.081	0.801
T30	-0.341	0.278	T30	-0.491	0.105
Body condition	r_s	*p*	Body condition	r_s	*p*
T0	-0.121	0.707	T0	0.186	0.562
T10	-0.421	0.173	T10	0.188	0.558
T30	-0.048	0.883	**T30 ***	**0.614**	**0.034**

* Exclusion of 2 outliers (2 females) resulted in the absence of significance (see also Figure 2.4).

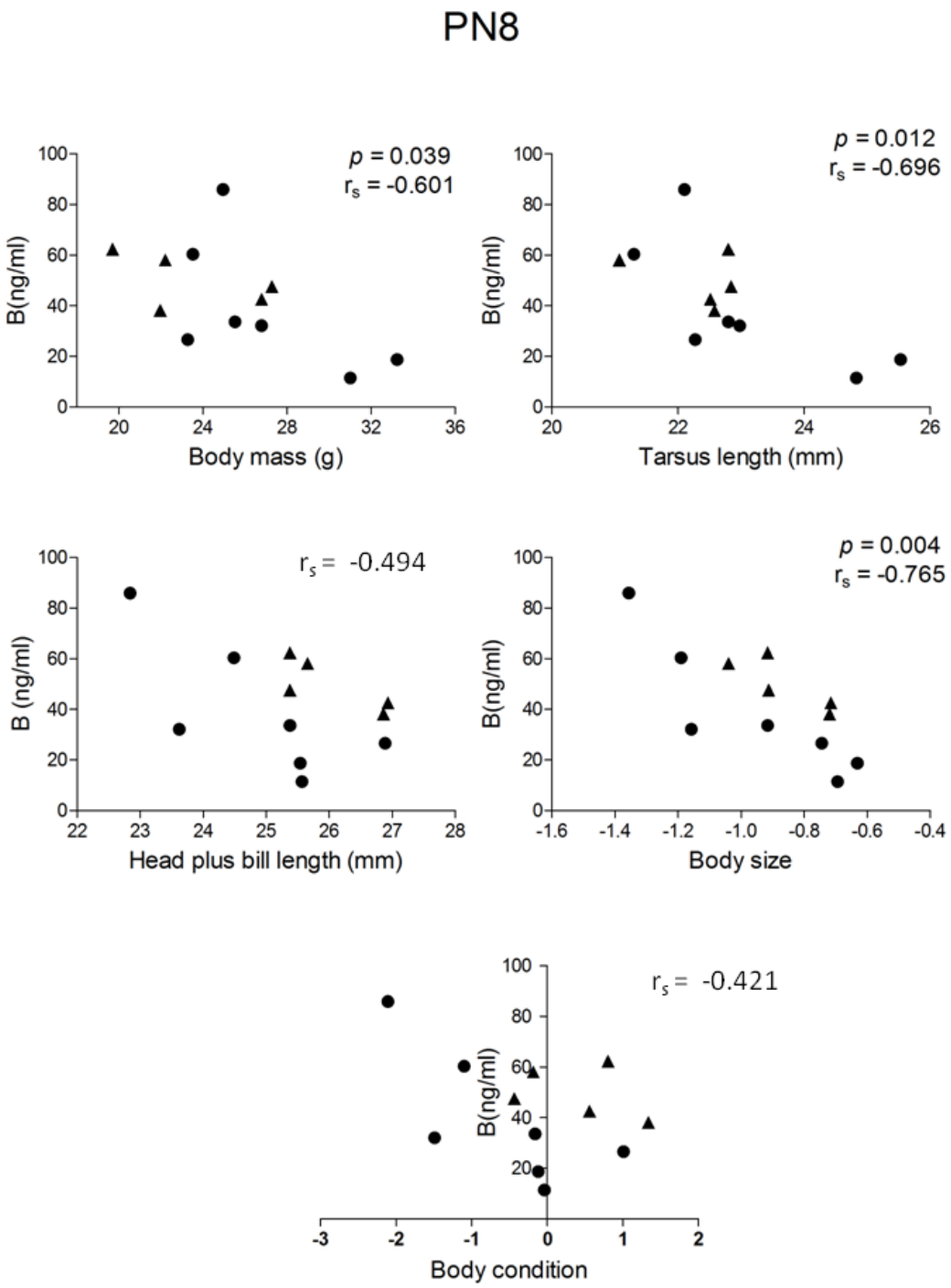

Figure 2.3 Morphometric traits (body mass, tarsus length, head plus bill length, body size) and energetic resources (body condition) associated with individual variation in stress-induced corticosterone (B) concentrations during a standardised restraint stress protocol 10 min following the capture in Japanese quail aged 8 days (PN8); circles: females; triangles: males; $p < 0.05$ denotes significant correlations; r_s = Pearson's coefficient of correlation.

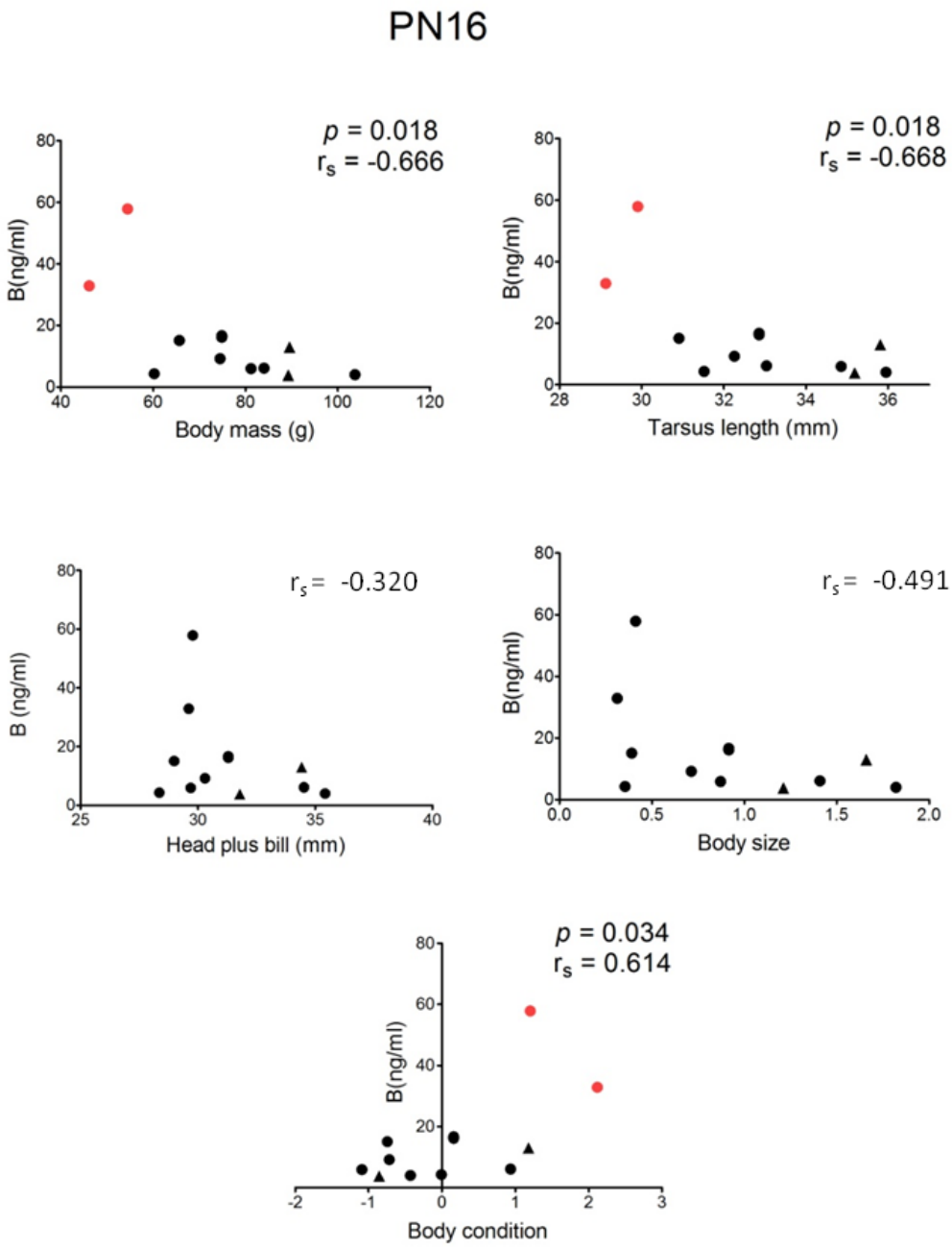

Figure 2.4 Morphometric traits (body mass, tarsus length, head plus bill length, body size) and energetic resources (body condition) associated with individual variation in stress-induced corticosterone (B) concentrations during a standardised restraint stress protocol after 30 min of the capture in Japanese quail aged 16 days (PN16). Circles: females; triangles: males; $p < 0.05$ denotes significant results; r_s = Pearson's coefficient of correlation. The removal of 2 females (statistical outliers highlighted in red) resulted in the absence of significance (see Paragraph 2.4.2 for details on the statistics).

2.5. Discussion

In this study I examined post-hatching B stress responses to a standardised environmental stressor and the potential links between B and (1) individual morphometry and (2) body condition in Japanese quail hatchlings aged 8 and 16 days. To the best of my knowledge, this study is the first attempt investigating the hypothesis of potential HPA axis-age-related differences across differing post-hatching stages of development in this avian model system. I point out, however, that in the present study I was unable to analyse potential sex-specific differences on the post-hatching development of the HPA axis because the data were female-biased.

The results from the present study suggest that, in the Japanese quail, the juvenile HPA axis responsiveness declines with post-hatching age, in accordance with the *"Developmental Hypothesis"* (e.g. Schwabl 1999; Blas *et al.*, 2006). Specifically, I found that the 8-day-old hatchlings showed higher maximal stress responsiveness over the 30-min restraint period than the 16-day-old hatchlings. As there were no significant differences in baselines between the two ages, the difference in the magnitude of the stress response is likely to be the result of the ontogenetic decrease in adrenocortical activity with post-hatching age. These findings support previous work in other precocial species (e.g. Holmes *et al.*, 1989; Dickens and Romero 2010). In contrast to these results, a gradual increase in the HPA responsiveness has been reported in non-precocial birds for which adrenocortical responses show higher or comparable adult-like activity patterns when the nestlings reach fledging (Sims and Holberton 2000; Love *et al.*, 2003; Walker *et al.*, 2005; Wada *et al.*, 2007). The results of this study suggest that there were no significant differences in the recovery trajectories to baseline (i.e. B change between T10 and T30) between the PN8 and PN16 young quail. Here, sample collection was based on prior published work in birds (including the Japanese quail), which indicates that a 30 min restraint period is an adequate protocol to analyse the overall shape of the stress response (i.e. maximum responsiveness and recovery to baseline) (Cockrem, 2013; Wall, 2010). It should be noted, however, that there was a high variation in the younger 8-day-old birds at T30. Future studies may wish to include more sampling times in

order to investigate in more detail potential age-related changes in the HPA axis negative feedback efficiency.

In an attempt to explore the *"Developmental Hypothesis"* and the links between the ontogeny of the HPA axis across the differing developmental strategies, it has been proposed that hatching in precocial species may be equivalent to fledging in non-precocious species (Wada, 2008). Consequently, the elevated stress responsiveness observed in precocial birds near hatching would correspond to the B peaks described near fledging in non-precocious species (Wada, 2008). The accelerated development of the adrenocortical activity in precocial birds compared to altricial species is also confirmed by studies performed in the precocial embryos. For example, studies in the chicken and mallard duck consistently showed that embryos have detectable endogenous baseline B concentrations and can exhibit a stress response after ACTH injection or painful stimuli from at least the second half of incubation (Wise and Frye, 1973; Hall, 1977; Scott *et al.*, 1981; Carsia *et al.*, 1987; Holmes *et al.*, 1990; Tona *et al.*, 2005). To the best of my knowledge, we lack studies on embryonic B secretion and HPA axis activity in altricial and semi-altricial species. However, as mentioned in the Introduction (Paragraph 2.1), the data available in non-precocious species post-hatching suggest that the nestlings have limited capacity to activate the adrenocortical response at least during the early nestling stages. Altricial nestlings are nest-bound and parent-dependent, with limited capacity to escape from environmental threats, such as predators. Therefore, a hypo-responsive HPA axis before reaching independence may have been evolved to prevent unnecessary rises in stress hormone concentrations, which would not help the chicks to move away from the challenge, but might negatively impact on the animal's growth (Blas *et al.,* 2006; Spencer *et al.,* 2009). Much less clear, however, is the adaptive significance underlying the decline in the HPA axis responsiveness with post-hatching age in precocial birds. Glucocorticoids have an important modulatory role in cognition both during adulthood and development (Sandi and Rose, 1994; McEwen and Sapolsky, 1995; Loscertales *et al.,* 1997). It has been proposed that elevated B concentrations in the first days after hatching in precocial birds may promote cognitive processes, including filial imprinting, learning events and social stress (Frigerio *et al.,* 2001). Moreover, precocial birds grow at slower rates than altricial birds (Lesage and Gauthier,1997). In this

study both 8- and 16-day old quail were clearly able to mount B stress responses, similarly as in other precocial species (Holmes *et al.*, 1989; Dickens and Romero 2010). Such capacity, which appears delayed in altricial species (e.g. Schwabl, 1999; Sims and Holberton, 2000; Wada *et al.*, 2007) might expose precocial birds to frequent repeated acute surges of endogenous B concentrations in response to daily stressors, and the metabolic effects of these elevated endogenous B levels may impact negatively on growth. This led me to speculate that in precocial birds the potential costs of having evolved an active/hyper-responsive HPA axis on growth patterns may be compensated by a higher investment in cognitive abilities, which may be more important for survival during the critical post-hatching time windows when the juveniles are likely to be more vulnerable to mortality. More work is needed to further explore the biological variation of the ontogeny of the stress system in order to improve our understanding of its evolutionary significance in vertebrate animals. The high degree of developmental strategy variation in birds across the precocial-altricial spectrum makes avian systems ideal models for furthering our understanding in this area.

In both 8- and 16-day old quail baseline B levels did not correlate with any morphometric measurements recorded. However, body mass and body size in the younger 8-day-old quail correlated with the variation in B concentrations at T10, with lighter and smaller individuals showing higher stress-induced levels. The negative correlation between stress-induced B at T10 and body size was driven by the tarsus length and not by the head plus bill length. These findings partially support my predictions and are in agreement with previous work showing negative relationships between body mass or structural size and stress-induced or chronically elevated B levels (Lesage and Gauthier, 1997; Saino *et al.*, 2005; Dickens and Romero, 2010). Surprisingly, none of the morphometric traits measured in the 16-day-old hatchlings significantly co-varied with B. I propose three non-mutually exclusive explanations for such potential age-related differences. First, there may be specific developmental windows, likely during the very early stages of post-hatching development, in which quail may be more plastic to adapt their body mass and shape in response to the quality of the living environment. An alternative possibility is that differences in the relationship between stress hormones and morphometry are the result of physiological constraints, for example due to metabolic demand differences

related to the size of the juvenile individuals rather to their age. In fact, as glucocorticoids often promote mobility (Breuner *et al.,* 1998a, b) it is plausible that the lighter and smaller juveniles may have had higher metabolic rates, which in turn were associated with enhanced HPA responsiveness. Thirdly, I cannot exclude the possibility of sex-specific differences in the regulation of the body mass and skeletal elements with the HPA axis responsiveness. For instance, there is the suggestion that males may be more susceptible than females to the effects of pre-hatching stress on growth (Love *et al.,* 2005; Hayward *et al.,* 2006; Love and Williams, 2008). The experimental groups in this study were female-biased, especially at PN16 when there were only 2 males compared with 10 females, meaning that I could not explore potential dependencies between the HPA axis and sex. Overall, I acknowledge that these data are correlative and therefore provide only a presumed link between the intensity of B stress responses and the individual's morphometry within specific developmental windows in the Japanese quail. I also point out that the lack of correlations between baseline B levels and the recorded morphometric traits may be the consequence of the limited sample size used in this study. Rigorous experimental manipulations across differing stages of development would be needed to disentangle the factors contributing to phenotypic variation and the intensity of the stress system in the juveniles. Such factors are likely to involve both active and passive regulatory processes modulating the adrenocortical secretion in response to environmental stressors. I also point out that baseline B levels should be a better indicator of morphometric and body condition rather than stress-induced B levels.

I did not find any significant correlation between B and the body condition at either age. Although a variety of studies carried out in adult bird species did find negative correlations between baseline or the magnitude of the stress response and body condition (e.g. Schwabl and Kriner, 1991; Gwinner *et al.,* 1992; Love *et al.,* 2005), the results from this study support the main trends emerging from the limited work conducted in juvenile birds (Romero *et al.,* 1998; Schwabl, 1999; Sims and Holberton, 2000; Love *et al.,* 2003). Furthermore, a recent study in which juvenile European starlings (*Sturnus vulgaris*) where chronically

exposed to stress during both the pre- and post-hatching developmental stages reported a negative correlation between stress-induced B levels and body condition in the stressed-juveniles but not in the controls (Love and Williams, 2008). Similarly, poor quality food and dietary restriction in back-legged kittiwake (*Rissa tridactyla*) juveniles lowered body energy reserves and enhanced baseline and HPA axis responsiveness (Kitaysky *et al.,* 1999). Altogether, these data suggest that prolonged, rather than temporary elevations of stress hormones may be involved in changes of the energetic conditions. Experimental manipulation in order to modify endogenous energy reserves in young individuals can be a valid experimental approach to test such a suggestion.

2.6. Conclusion

The results from this study showed that the juvenile stress reactivity in Japanese quail declined with post-hatching age. These data concur with those found in the few other studied precocial species and are in accordance with the *"Developmental Hypothesis"*. Moreover, the results confirm the presence of relevant links between circulating glucocorticoid stress hormones and the individual's morphometry (i.e. body mass and structural size), but also suggest that such associations vary throughout development. The limited sample size in this study constrained my ability to examine sex-specific changes in the development of the HPA axis post-hatching and future studies with larger number of birds are needed to examine this relevant aspect. Moreover, future research investigating the ontogeny of the adrenocortical responses across a wider developmental window, ideally starting from the pre-hatching stages, will be extremely useful to test predictions between potential age-related differences and the species's developmental mode. The large variety of developmental strategies in birds across the precocial-altricial spectrum makes this taxon a reliable model to study HPA axis development in the context of life-histories trade-off variation and to link it with the evolutionary mechanisms of stress physiology among vertebrate species.

3. Chapter

Pre- and post-hatching stress in context: effects on the post-hatching stress physiology in growing and adult Japanese quail

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3.1 Abstract

Developmental stress can significantly influence physiology and survival in many species. Mammalian studies suggest that pre- and post-natal stress can have different effects (i.e. hyper- or hypo-responsiveness) on the hypothalamic–pituitary–adrenal (HPA) axis. In mammals, the physiological intimacy between mother and offspring constrains the possibility to control, and therefore manipulate, maternal pre- and post-natal influences. Here, using the Japanese quail as study species, I elevate levels of the glucocorticoid stress hormone corticosterone (B) *in ovo* and/or in the endogenous circulation of juveniles. I examine the effects of treatments on B, glucose, glycerol and triglyceride stress responses at two different ages, in juvenile and adult quail. In juveniles, B data reveal a sex-specific effect of post-hatching treatment regardless of the previous pre-hatching protocol, with post-hatching treated females showing attenuated stress responses (i.e. quicker return to baselines) in comparison with the other groups, while no differences are observed among males. In adulthood, the birds that hatched from eggs in which yolk B levels were experimentally elevated show higher B concentrations over the stress response compared with controls. This effect is not evident in birds subjected to either post-hatching treatment or the combined treatments. There are no effects on glycerol or glucose in the

juveniles. However, post-hatching B manipulation induces short-term alterations in basal triglyceride concentrations in the juveniles, which are linked with sex and basal glucose concentrations of the birds; whilst pre-hatching B treatment induce long-term alterations on basal glucose and these effects, similarly as before, interact with sex. These results demonstrate that (1) early glucocorticoid exposure can have both transient and long-term effects on the HPA axis, depending upon the developmental stage and sex and (2) elevated endogenous B levels post-hatching can modulate the effects induced by exposure to elevated B pre-hatching on the HPA activity.

3.2 Introduction

Environmental cues during the sensitive periods of early life can shape developmental trajectories and influence a wide range of phenotypic traits later in life (Mousseau and Fox, 1998; Monaghan, 2008). A considerable number of studies have investigated to what extent developmental stress can modulate endocrine systems and influence adult health outcomes (Ward, 1972; Barker *et al.*, 1990; Gluckman *et al.*, 2007). In vertebrates, environmental stressors such as food shortages or extreme weather can activate the Hypothalamic-Pituitary-Adrenal axis (HPA axis; Wingfield, 1994; Breuner *et al.*, 1998b; reviewed by Romero, 2004). This activation leads to a short-term surge of glucocorticoids, which mobilise energy resources and divert behaviour to life-saving strategies (Wingfield *et al.*, 1998). In the long-term, however, elevated stress hormones can compromise HPA axis functioning and can have negative implications for the nervous and immune systems, body energy balance and redox physiology (McEwen and Stellar, 1993; Sapolsky, 2000; de Kloet *et al.*, 2005a; Costantini *et al.*, 2011a). There is increasing evidence to suggest that if laying/gravid females experience stressful stimuli, which elevate endogenous glucocorticoids (e.g. predation pressure, social instability, unpredictable feeding or direct glucocorticoid exposure), their embryos can also be exposed to these circulating stress hormones through the placenta (Seckl, 2004; Kaiser and Sachser, 2005) or their presence in the egg (fish: McCormick, 1999; reptiles: De Fraipont *et al.*, 2000; Meylan *et al.*, 2002; birds: Hayward and Wingfield, 2004; Love *et al.*, 2005; Saino *et al.*, 2005). Similarly after birth, post-natal/post-hatching

stressors such as sibling competition, low food provisioning, maternal deprivation or direct glucocorticoid administration can lead to an increase in endogenous glucocorticoids in the offspring (reptiles: Meylan *et al.*, 2002; birds: Kitaysky *et al.*, 1999; Love *et al.*, 2003; Spencer *et al.*, 2003; Spencer *et al.*, 2009; mammals: Rosenfeld *et al.*, 1992; Fey and Trillmich, 2008). Recent research in a variety of vertebrate taxa has shown that early life glucocorticoid manipulations can influence a wide range of phenotypic traits, including growth (Spencer and Verhulst, 2007; Saino *et al.*, 2005), metabolic rate (Sloman, 2010), stress-related behaviours and cognitive performances (Vallée *et al.*, 1997; Vallée *et al.*, 1999; De Fraipont *et al.*, 2000; Meylan *et al.*, 2002; Rubolini *et al.*, 2005; Spencer and Verhulst, 2007; Sloman, 2010; Boogert *et al.*, 2013), and can suppress survival chances into adulthood (Monaghan *et al.*, 2012).

It has been suggested that the organisational role of developmental exposure to stress hormones on the phenotype is likely to be caused by changes in HPA axis activity that modulate sensitivity to environmental stressors later in life (recently reviewed by Harris and Seckl, 2011). Pioneering studies in mammalian models (primarily rodents), suggest that the effects of pre-natal stress on the offspring HPA axis may be different from those caused by post-natal stress. In fact, while maternal pre-natal stress often results in HPA hyper-responsiveness, with pre-natally stressed offspring exhibiting enhanced and prolonged stress hormone release in response to stress (Henry *et al.*, 1994; Barbazanges *et al.*, 1996; Kapoor *et al.*, 2006); post-natal exposure to stressors, such as "neonatal handling", can produce dampened stress responsiveness (Levine *et al.*, 1967; Meaney and Aitken, 1985; Vallée *et al.*, 1996; Liu *et al.*, 1997; Macrì *et al.*, 2004). Importantly, several post-natal manipulations in rat pups are known to cause changes in the amount of maternal care provided by the dams, which to a certain degree, can buffer or counteract the effects of previous pre- and post-natal stressors (Maccari *et al.*, 1995; see also review by Macrì and Würbel, 2006). On one hand, these data raise the question, surprisingly understudied, of interactive influences between pre- and post-natal experiences. However, they also draw attention to the difficulties in determining whether the observed effects are mediated by altered maternal HPA axis, by direct changes in the offspring HPA reactivity, or by an interaction of both as recently proposed (Macrí and Würbel, 2006). Birds offer advantages over mammalian species to

experimentally manipulate pre- and post-hatching environments, minimising interactions with the mother's physiology (reviewed by Henriksen *et al.*, 2011 and Schoech *et al.*, 2011; see also Love and Williams, 2008; Spencer *et al.*, 2009). Precocial birds in captive conditions can be reared without post-hatching maternal contact, thereby excluding the potential confounding actions of maternal care. Furthermore, avian and mammalian neuroendocrine systems are highly conserved (Wingfield, 2005a), facilitating comparative approaches in a more evolutionary framework (Groothuis *et al.*, 2005).

The few studies conducted in birds to date have demonstrated that pre- (Hayward and Wingfield, 2004; Hayward *et al.*, 2006; Love and Williams, 2008; Haussmann *et al.*, 2012) or post-hatching stressful conditions (Love and Williams, 2008; Spencer *et al.*, 2009) can lead to long-term effects on the HPA axis physiology. However, more studies are needed to fully understand the directions of these modifications as they are likely to differ across bird species and life stages. To this end it is important to consider both pre- and post-hatching contexts (Love and Williams, 2008; Monaghan, 2008). Furthermore, there is little information in birds about the links between early life stress and changes in metabolic energy expenditure (Spencer and Verhulst, 2008), and to what extent they are linked with HPA axis modifications. High glucocorticoids in early life may induce changes in metabolic responses that can help juveniles to deal with stressful circumstances in the short-term, but can have costs into adulthood (Gluckman *et al.*, 2007; Cottrell and Seckl, 2009). The stress system has a key role in the control of glucose transport: in several vertebrate species acute stress can increase circulating glucose concentrations (Curi *et al.*, 1990; Widmaier and Kunz, 1993; Carragher and Rees, 1994; Remage-Healey and Romero, 2000, 2001). At the same time, such an increase in available energy may be enhanced by the activation of the breakdown of plasma triglycerides into glycerol and free-fatty acids (Remage-Healey and Romero, 2001). Although it is known that the prolonged elevation of glucocorticoids can cause changes in glucose and lipid metabolism (Norris, 1997), the effects of experimentally elevated stress hormones during early life on these metabolites have received little attention.

Chapter 3

The main aim of the present study was to analyse whether, and the extent to which, exposure to stress hormones during differing developmental stages would influence the HPA system and its related metabolism in the Japanese quail. Specifically, the objectives of this study were (i) to analyse if the exposure to physiological stress hormone levels during pre- and post-hatching development would cause changes in the dynamics of the stress responses of B (corticosterone, the main glucocorticoid in birds) and plasma metabolites involved in glucose transport and lipid metabolism in the short- and long-term and, (ii) to examine potential short- and long-lasting interactive effects between pre- and post-hatching stressful stimuli. To accomplish such objectives, I mimicked a prolonged exposure to physiological stress through direct manipulations with B *in ovo* and/or in the juvenile quail. I then performed a standardised environmental stress test (Wingfield *et al.*, 1982) at two distinct post-hatching stages, in juvenile and adult non-breeding quail, and measured responses to stress of B, glucose, glycerol and triglycerides. I also monitored growth rates to assess direct *vs.* indirect effects of developmental B on the stress responses (Metcalfe and Monaghan, 2001; Spencer *et al.*, 2009). It has been suggested that precocial birds may be especially sensitive to poor/stressful environmental conditions experienced pre-hatching (Metcalfe and Monaghan, 2001). In fact, precocial birds show larger embryonic growth rates and larger brain masses at hatching than altricial birds, which, on the opposite, have their major period of morphological, as well as neuroendocrine and neural development (i.e. cell proliferation and differentiation, synapse formation and myelination) post-hatching (Rogers, 1995; Starck and Ricklefs, 1998). Based on these relevant developmental differences between altricial and precocial bird species, it appears reasonable to predict that in the Japanese quail pre-hatching exposure to B would produce a stronger and longer-lasting impact than post-hatching exposure to B. I tested this prediction by comparing the effects of pre-hatching B, post-hatching B and their combined effect on the HPA axis physiology and its related metabolism.

3.3 Material and methods

3.3.1 Experimental design

The animal work was conducted at the Cochno Farm and Research Centre, University of Glasgow, UK. All indoor rooms were climate controlled at 19°C on a 12:12-h light-dark cycle (lights on 7am-7pm). Eggs used in this experiment were obtained from this breeding stock. Breeding quail (n = 20 females, 10 males) were housed in trios (2 females:1 male) in 79 X 48 X 58 cm enclosures that were maintained throughout the experimental period (September 2010-March 2011). Fresh-laid eggs were collected, identified by colour and pattern and marked according to maternal identity. Four groups of experimental birds were established and treated as follows: 1. pre-hatching and post-hatching untreated birds (CC); 2. pre-hatching B-treated and post-hatching untreated birds (BC); 3. pre-hatching untreated and post-hatching B-treated birds (CB); 4. pre-hatching B-treated and post-hatching B-treated birds (BB). Treatment order was counterbalanced across females. The experiment was repeated twice (batch 1: September 2010-December 2010; batch 2: December 2010-March 2011).

3.3.1.1 Pre-hatching environment and pre-hatching hormonal manipulation

The eggs were incubated at 37.5^{o}C and 55% humidity while being turned twice hourly (incubator Ova-Easy 190A, Brinsea Products Ltd, UK). The day on which incubation started was designated as embryonic day 0 (E0). At day E5, fertile eggs were identified using a bright light source and selected for the yolk hormonal manipulation with B. The eggs were then injected at the conical tip with 10µl of a sterile solution of B (Sigma Aldrich, Poole, UK; concentration B: 850 ng/ml) dissolved in peanut oil (B-eggs; n = 74) or with 10µl of sterile peanut oil alone (C-eggs; n = 74) using a SGE syringe (Fisher Scientific, Loughborough, UK). Here, E5 rather E0 (e.g. Hayward *et al.*, 2006) was chosen as this is the point at which it is possible to reliably determine egg fertility in the Japanese quail. Injection prior to this point would have meant that I might inject non-fertile eggs, which will not develop and hence artificially inflate the perceived

number of animals used in the study. Importantly, in birds the egg yolks are stratified in layers at laying and hormone concentrations, including B, differ among these layers (e.g. Lipar *et al.*, 1999; Almasi *et al.*, 2012). Yolk layers break down after a few days of incubation with the yolk becoming mixed. The injection protocol used here, therefore, ensured that B levels were elevated once yolk layers have ceased to exist. Pilot dye studies were carried out prior to the experiment to determine the depth of injection required to place the hormone into the yolk (Karen Spencer's personal communication). The dose of B injected (8.5ng) was designated to elevate endogenous B concentrations within the yolk by 1.8x Standard Deviation (SD) of the mean above control eggs, similar to previous studies in birds (Rubolini *et al.*, 2005; Saino *et al.*, 2005; Hayward *et al.*, 2006; Love and Williams, 2008). This physiological increase was confirmed to be within the relevant biological levels by previous pilot work that quantified yolk B concentrations in a sample of eggs (n = 8) taken from a previous generation of our breeding females using both Radioimmunoassay and Liquid Chromatography-Mass Spectroscopy (Boogert *et al.*, 2013). Needle punctures were then sealed with a transparent and breathable wound dressing (Germolene New Skin, UK). As soon as the injected area appeared dry, the egg was returned to the incubator. At day E14 eggs were transferred into hatchers within the same incubator, and humidity was increased to 70-75%. In each hatcher, eggs were separated according to maternal identity with plastic dividers so that the identity of the birds could be determined post-hatching.

3.3.1.2 Post-hatching environment and post-hatching hormonal manipulation

Upon hatching (between days E17-E19; hatch rates averaged 61.1% and there were no significant differences in hatching success between C-eggs and B-eggs, t-test = 2.0, p = 0.2), quail were labelled with unique colour combinations using nail varnish, weighed to the nearest 0.01g (hatching mass, day PN0) using a balance (Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, UK) and placed back into the hatcher to allow the plumage of the birds to dry.

Subsequently, quail hatched from B-eggs were assigned to either the BC treatment (final n: female = 10, male = 6) or the BB treatment (final n: female = 9, male = 9); quail hatched from C-eggs were assigned either to the CC treatment (final n: female = 9, male = 14) or CB treatment (final n: female = 10, male = 10). After 24-36 hours post-hatching (day PN1), the birds were weighed again and housed in 4 different treatment-specific enclosures in a single room (in the second batch treatment-specific enclosure positions were reversed to control for an enclosure effect). Food (turkey starter crumbs, Dodson and Horrell, Northamptonshire, UK) and water were available *ad libitum*. A brooding lamp was placed over each enclosure to ensure an initial brooding temperature of 35.5^{o}C for the first 3 days of age (from day PN3 temperature declined daily by 1-1.5^{o}C until day PN19 when warming bulbs were switched off and the birds were subjected to the ambient temperature of 19^{o}C). Enclosures were each divided into 2 or 3 compartments with cardboard dividers so that juveniles of the same age were housed in the same compartment (n = 2 to 7).

Between days PN5-19, birds in the CB and BB treatments were subjected to oral supplementation with B, while birds in the CC and BC were given carrier alone using mealworms injected with B (*Tenebrio molitor*, size 13-18mm) (Breuner *et al.*, 1998a). To ensure the birds would ingest mealworms they were provided with un-injected mealworms for 3 days prior to the experimental manipulations. During the oral B manipulation period, mealworms were removed from the fridge and injected with 10µl of B solution dissolved in peanut oil (concentration B: 4.5mg/ml between days PN5-15 and 9mg/ml between days PN16-19) or 10µl of peanut oil using a syringe (Hamilton, UK). To confirm that each bird was eating one single mealworm per day, juveniles within the same brooder compartment were separated with transparent dividers during feeding. Generally mealworms were fully ingested within the first 5-10 minutes (and always within 18min). To ascertain that the post-hatching manipulation was physiological and mimicked a standardised acute stressor, I carried out pilot work prior to the start of the experiment. I first measured the natural variation of acute stress responses in a group of birds from a previous generation of the breeding stock at PN8 (n = 12) and PN16 (n = 12). The results from this study are reported in detail in Chapter 2. On the basis of the former data and the literature in birds (Hull *et al.*, 2007; Spencer and Verhulst, 2007; Spencer *et al.*, 2009; Wall and Cockrem, 2009), I

then performed a second experiment and tested the effects of two different B doses, a high-B dose of 0.45mg and a low-B dose of 0.045mg in 4 independent groups of birds at day PN8 and PN16 (high-B, N = 5; low-B, N = 4 at both ages). I administered a single oral dose to each individual bird using injected mealworms as described above and took a blood sample 10min post-B supplementation. The low-B dose at day PN8 elevated plasma B levels by 1.8x SD of the mean above the 10-min B peak determined previously, while at day PN16 there was no significant change in plasma B levels. The high-B dose was supra-physiological at both ages. For the current study, I therefore scaled the hormonal dose to produce a daily physiological 10-min B peak for each age interval (e.g. Spencer *et al.*, 2009): 0.045 mg/day between days PN5-15 and an intermediate B dose of 0.09mg/day between days PN16-19. During the present experiment, I tested whether the B dose of 0.09mg/day was biologically relevant by sampling a sub-sample of birds at day PN16 as described above. Plasma B levels in CB and BB birds were found to be similar to the 10 min-B peak observed in the first pilot study at PN16 (n = 11, pooled data: mean ± s.e.m., 18.77 ± 4.55ng/ml).

At day PN19 juveniles were sexed by sexual dimorphic plumage and singly housed in 61 X 46 X 51cm enclosures, in visual and auditory contact with conspecifics. Post-natal mortality rates averaged 8.1%.

3.3.2 Analysing the short- and long-term effects of pre- and post-hatching B exposure

3.3.2.1 Growth

Body mass was measured to the nearest 0.01g at regular intervals until day PN64; from day PN3 onwards tarsus length and head plus bill length were also measured to the nearest 0.1mm with a digital calliper (Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, UK). All the body measurements were taken only by myself to minimise technical variations. Blind measurements to bird treatment could not be achieved as birds were housed in treatment-specific brooders until PN19.

3.3.2.2 Standardised capture-restraint-stress protocol

Acute stress responses were measured using a standardised capture-restraint protocol on days PN22 and PN64. I chose PN22 (3 weeks of age) and PN64 (9 weeks of age) because I wanted to test the effects of developmental B exposure in the short-term soon after the end of the post-hatching treatment and over the long-term in non-breeding sexually mature individuals, respectively. In fact, Japanese quail reach puberty between 6-8 weeks (Ottinger, 2001). Therefore the quail sampled at PN64 were fully grown and capable of breeding if they would have been stimulated with an appropriate reproductive induction protocol (Robinson and Follett, 1982). Birds were removed from their cages between 09:15 and 12:40h and a basal blood sample (T0) was collected within 2 (mean ± s.e.m, 1.43 ± 0.04) min of opening the cage (Wingfield *et al.*, 1982). Each bird was then placed into an opaque box (14.5 X 13.5 X 14.5 cm) and further stress-induced blood samples were taken after 10 (mean ± s.e.m, 10.52 ± 0.08) min and 30 (mean ± s.e.m, 30.31 ± 0.06) min of opening the cage (T10 and T30, respectively). 20µl of T0 and T30 samples were immediately used to measure glucose concentrations using a glucose meter (GlucoMen Visio, Manarini Diagnostics, Firenze, Italia). T0 and T30 glucose samples were chosen to represent basal and stress-induced glucose levels, respectively (e.g. Curi *et al.*, 1990; Carragher and Rees, 1994; Remage-Healey and Romero, 2000, 2001). Each batch of glucose strips (n=50) comes with an individual barcode. For each glucose meter barcode used, I estimated the intra- and inter-assay coefficients of variation by measuring normal and high quality control solutions provided by the manufacturer. The intra-assay variation for normal and high quality controls was 3.25% and 3.18%, respectively. The inter-assay variation for normal and high quality controls was 4.34% and 1.81%, respectively. Once the stress response protocol was concluded, body mass, tarsus length and head plus bill measurements were taken for each bird. Remaining blood samples were kept on ice for up to 4h before being centrifuged and plasma aliquots withdrawn and stored at -20^{o}C.

3.3.2.3 B Radioimmunoassay

B was extracted from 10- to 30-µl plasma samples (mean ± s.e.m., 21.81 ± 0.15µl plasma) and measured by Radioimmunoassay using the protocol described in Chapter 2, Section 2.3.2. Extraction efficiencies averaged 93% ± 0.003 s.e.m. B samples from the same individuals were analysed in the same assay and samples from different treatments were randomised among the assays (n = 3). The intra-assay coefficients of variation were 10% and 20% and 23%, while the inter-assay variation at 80%, 70% and 50% binding were 17%, 18% and 9%, respectively.

Table 3.1 Percentage of undetectable corticosterone (B) samples across the treatment groups at post-hatching (PN) day 22 and 64 during a standardised capture-restraint protocol within 2, 10 and 30 min (T0, T10 and T30, respectively) of opening the cage.

Day PN22:	Treatment			
Time	CC	BC	CB	BB
T0	95.7	68.8	80.0	83.3
T10	47.8	12.5	45.0	22.2
T30	56.5	37.5	65.0	61.1

Day PN64:	Treatment			
Time	CC	BC	CB	BB
T0	73.9	53.3	70.0	72.2
T10	56.5	12.5	35.0	50.0
T30	34.8	18.8	50.0	55.6

Unexpectedly, concentrations of B in 53.7% of the samples were undetectable. In fact, it should be noted that plasma B concentrations were much lower than in the previous study (Chapter 2). This was not due to technical problems with the Radioimmunoassay itself as the quality controls, which were the same as the quality controls used in the previous study (Chapter 2), were within the expected concentration range. Therefore the large number of undetectable samples represented a true biological effect, likely consequence of the frequent handling of the birds for taking morphological measurements as discussed in

Paragraph 3.5.1. Preliminary chi-square tests showed that the likelihood of encountering undetectable values differed significantly across the stress responses. At both days PN22 and PN64, the highest percentage of undetectable values was observed in the T0 samples (day PN22: $X^2 = 38.53$, $df = 2$, $p < 0.0001$; T0 = 83.1%, T10 = 33.8%, T30 = 55.8%; day PN64: $X^2 = 16.76$, $df = 2$, $p < 0.0001$; T0 = 68.8%, T10 = 40.3 %, T30 = 40.3 %). I then performed additional chi-square tests at each time of sampling to test for a potential treatment effect. At day PN22 there was no effect of treatment in the T0 and T30 samples (T0: $X^2 = 5.07$, $df = 3$, $p = 0.17$; T30: $X^2 = 3.07$, $df = 3$, $p = 0.38$), while there was a tendency in the T10 samples ($X^2 = 7.47$, $df = 3$, $p = 0.06$) due to a lower percentage of undetectable levels in the BC and BB groups (Table 3.1). Similarly, at day PN64 there was no effect of treatment in the T0 and T30 samples (T0: $X^2 = 1.57$, $df = 3$, $p = 0.67$; T30: $X^2 = 5.90$, $df = 3$, $p = 0.12$). At T10, I found a significant treatment effect ($X^2 = 8.59$, $df = 3$, $p = 0.03$) due to a lower percentage of undetectable samples in the BC adult quail (Table 3.1). In order to investigate in a further statistical model these potential treatment differences (see paragraph below), I set undetectable B concentrations to the individual detection limits of each sample, calculated according to the individual extraction efficiencies and plasma volumes (mean ± SEM, 1.64 ± 0.29ng/ml), as shown in Landys *et al.*, (2010). This approach provides the most conservative estimate for statistical comparisons.

3.3.2.4 Glycerol and triglyceride assays

Glycerol and triglyceride levels were measured using the Serum Triglyceride Determination kit (Sigma-Aldrich, Dorset, UK). This assay has a two-step reaction sequence: it first measures free (i.e. unbound) plasma glycerol levels and then plasma triglyceride levels by using a lipoprotein lipase to cleave the triglyceride to glycerol and free fatty acids. In both the reactions, the glycerol is measured by colorimetric spectrophotometry at 540 nm. Consequently, the assay allows correcting all triglyceride measurements for the initial free glycerol measurements, which correspond to the “true triglyceride” (herein referred as “triglyceride”) concentrations. The lack of this correction has been shown to

result in an overestimation of circulating triglyceride concentrations (Howdieshell *et al.*, 1995).

In this study, I adapted the assay to 96-well plate readers (Corning Life Sciences, Amsterdam, The Netherlands) by scaling down the volume of the assay reagents, glycerol standard (concentration: 0.26mg/ml, Sigma-Aldrich, Dorset, UK) and plasma samples of 5 fold. This allowed me to reduce the volume of plasma samples to 2µl. To validate the assay, I first analysed the kinetics of the two reactions by incubating the reference (or blank), standard and plasma samples (T0 or T30) taken from a random sub-set of birds at PN22 or PN64 (n = 10) in a plate reader (Thermo Scientific Multiskan Spectrum, ThermoFisher, Vantaa, Finland) at 25ºC for 30min (incubation started immediately after adding the reactive reagent provided in the kit). I set the program in the plate reader in order to take the readings every min over the incubation period. Both the reactions showed their asymptote and stability at 10-15min of incubation. Therefore for the later analyses, I choose to take a single reading at 15min of incubation following the manufacturer's recommendations. Secondly, I made up 2 plasma pools to be used as quality controls for the later analyses. These pools gave absorbance values at approximately 0.4 and 0.1, corresponding respectively to a normal and low absorbance relative to the previously measured quail samples (herein referred as normal and low quality controls, respectively). Thirdly, I generated a glycerol standard curve (assay buffer PBS) to confirm that the absorbance was proportional to the known glycerol concentrations and a good parallelism with the previously measured quail samples. Finally, I estimated the intra- and inter-day plate variations using two plates over two different days for the reference and glycerol standard, which were on average within 10%.

After these preliminary tests, I performed the analyses with all the experimental samples. Samples from the same individual were measured in the same plate and samples from the different treatment groups were randomised across plates (n = 7). I measured glycerol and triglyceride concentrations in the T0 and T30 samples, which are meant to represent, respectively, basal and stressed-induced levels (Remage-Healey and Romero, 2001). I was unable to perform the analyses on 7 samples at day PN22 (T0: males, CC = 2; BC = 1, CB = 1, BB = 2; T30:

female, CC = 1) and 3 samples at PN 64 (T0: males, BC = 1; CB = 1, and BB = 1) because of the lack of plasma (B Radioimmunoassay was performed beforehand). As the triglyceride assay is linear up to 10mg/ml, the samples in which concentrations were above this value (i.e. almost all the females at PN64) were re-measured after being diluted in PBS buffer (dilution factors ranged from 1:2 to 1:5 according to the initial concentration measurements). The glycerol and triglyceride concentrations values were then calculated according to the reference and standard absorbance values as indicated by the Manufacturer. The intra-plate coefficient of variation for the normal and low quality controls was respectively 2.1% and 1.2% for the glycerol analyses and 5.8% and 4.5% for the triglyceride analyses. The inter-plate coefficient of variation for the normal and low quality controls was respectively 10.9% and 6.0% for the glycerol analyses and 6.6% and 5.5% for the triglyceride analyses.

3.3.3 Statistical analysis

Data analysis was performed in PASW statistics, version 19 (SPSS, Inc., 2009, Chicago, IL, www.spss.com) using Linear Mixed Effect models (LMEs) fitted by Restricted Maximum Likelihood or Generalized Linear models (GLMs). To meet the assumptions of the LME, response variables were transformed for normality when needed, all model residuals were normally distributed. Fixed factors were treatment, sex and their interaction; while batch and maternal identity were entered as random factors.

The growth curve between days PN1-36 was split into three discrete age intervals: days PN1-3, days PN8-19 and days PN22-36, which corresponded to periods before, during and after the post-hatching B treatment, respectively. For each interval, I estimated individual body mass growth rates by calculating the slope of a linear regression fitted for each bird. Likewise, I determined tarsus and head plus bill growth rates between days PN8-19 and between days PN22-36. These two measures of skeletal growth were transformed into a unique body size growth index by extracting the first component scores from a Principal Component Analysis (PCA) in each age interval (PCA days PN8-19 (PCA1): eigenvalue = 1.27, total variance = 63.44%; PCA days PN22-36 (PCA2): eigenvalue

= 1.26, total variance = 63.24%). Similarly, the first component scores from a PCA on tarsus and head plus bill absolute values measured at day PN64 (PCA day PN64 (PCA3): eigenvalue = 1.38; total variance = 69.15%) gave a body size index. Hatching mass, body mass growth rates and body mass at day PN64 were analysed in separate LMEs to disentangle potential short-and long-term effect of developmental B exposure. When needed, hatching mass values or the appropriate PCA was added into the LME as covariate (Table 3.2).

B, glucose, glycerol and triglyceride data were split by age (day PN22 and PN64) as I was specifically interested in examine short- and long-term effects of B on these physiological parameters. Except for B stress responses, basal levels (T0) and the response to stress (delta: the change in glucose, glycerol or triglyceride concentrations between T0 and T30) were analysed in separate models. In the LMEs for the glucose data, the glucose strip barcode was included as an additional random factor. Glucose and glycerol concentrations at day PN22 and PN64, as well as triglyceride concentrations at day PN22 were $\log_{10}$-transformed. Basal triglyceride levels and delta triglycerides at day PN64 could not be normalised because they were highly skewed (-1.8 < skewness < 1.5) and the data were analysed using GLMs with a gamma probability distribution and log-link function without the inclusion of random factors (the models with their inclusions were not resolvable).

As plasma triglycerides are well known to be affected by glucose metabolism through the insulin signalling pathways (Saltiel and Kahn, 2001), in a preliminary analyses I included glucose or delta glucose as covariate in the models performed for basal or delta triglyceride data, respectively. Except for the analysis of basal triglycerides at PN22, the inclusion of glucose did not alter the statistical outcomes obtained from the models performed without glucose, nor did glucose co-varied significantly with any of the variables ($p > 0.05$). Here, therefore, I reported the results from the full models without glucose apart from basal triglyceride analysis at PN22. B concentrations were inverse-transformed and the HPA responsiveness was analysed using similar LME as for the previous analyses with the addition of a repeated measure approach, to examine changes in B levels over the time of sampling (i.e. T0, T10, and T30). I included the interactions that were biologically meaningful to the study design: treatment x

time of sampling; treatment x sex, treatment x time of sampling x sex. In all the models, non-significant effects ($p > 0.05$) were dropped using a backward procedure following Crawley (1993). Post-hoc analyses for main effects were performed using the available Bonferroni method in PASW, which applies an adjustment to *p*-values to account for multiple comparisons. Significant interactions were further investigated in separate models using pre-hatching and post-hatching treatment as two distinctive fixed factors, each of them with two levels (C = control or B = corticosterone exposure). Unless otherwise specified, data are presented as mean ± s.e.m.

3.4 Results

3.4.1 Effects of pre- and post-hatching B exposure on growth

Hatching mass did not differ across treatments, sex or their interaction (Table 3.2a, Figure 3.1). Similarly, there were no treatment differences in body mass growth up to day PN3; there was no effect of sex, or hatching mass on growth (Table 3.2b, Figure 3.1). During the post-hatching B manipulation (days PN5-19), I found no differences in growth between B-treated and control birds. There was a significant positive co-variation between body mass growth rates and PCA1, but the slopes did not differ across groups (Table 3.2c; Figure 3.1). Once post-hatching B exposure had ceased, neither treatment nor its interaction with sex were significant for growth rates between days PN22-36 or for body mass at day PN64 (Table 3.2d-e; Figure 3.1); there was a significant effect of sex on both variables (Table 3.2d-e), with females showing larger growth and body mass than males. Also, growth between days PN22-36 and body mass at day PN64 co-varied positively with PCA2 and PCA3, respectively, but I found no other effects of these variables (Table 3.2d-e).

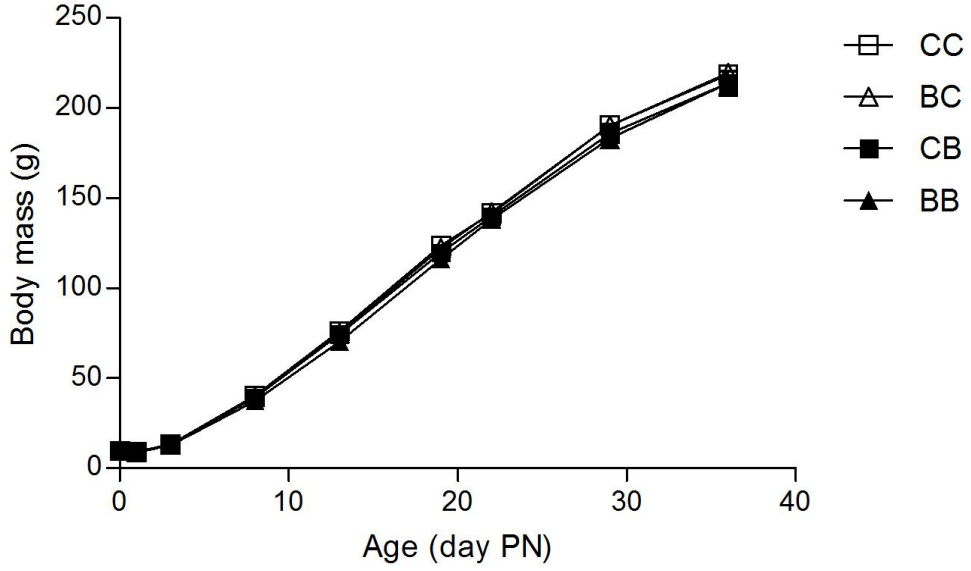

Figure 3.1 Increase in the body mass in the four treatment groups (i.e. CC, BC, CB and BB) during the first 36 days of post-hatching (PN) life. Sample sizes: CC = 23; BC = 16; CB = 20; BB = 18. Data represent means ± s.e.m.

Table 3.2 Results of Linear Mixed Effect modelling (LMEs) of potential short- and long-term effects of treatment, sex and their interaction on measures of post-hatching (PN) body mass or growth rates at day (a) PN0, (b) days PN1-3; (c) days PN8-19, (d) days PN22-36 and (e) day PN64. At days PN1-3, days PN8-19, days PN22-36 and day PN64, the appropriate covariate (hatching mass, PCA1, PCA2, PCA3, respectively) and its interactions with treatment and sex were included to control for body size. Superscripts: * denotes excluded factors during the stepdown procedure, numbers refers to the order of removal. In bold, significant factors ($p < 0.05$).

(a) Day PN0: hatching mass	d.f.	F	*P*
treatment	3, 58.05	1.54	0.21
sex * [2]	1, 59.66	0.21	0.65
treatment x sex *, [1]	3, 54.78	1.29	0.29
(b) Days PN1-3: body mass growth	d.f.	F	*p*
treatment	3, 66.64	0.22	0.88
sex *, [5]	1, 69.24	0.47	0.49
hatching mass *, [6]	1, 44.65	0.97	0.33
treatment x sex *, [3]	3, 60.08	0.68	0.68
treatment x hatching mass *, [2]	3, 57.48	0.19	0.90
sex x hatching mass *, [4]	1, 65.07	2.19	0.14
treatment x sex x hatching mass *, [1]	3, 56.40	0.84	0.48
(c)Days PN8-19: body mass growth	d.f.	F	*p*
treatment	3, 58.38	2.28	0.09
PCA1	1, 65.44	19.29	< 0.0001
sex *, [5]	1, 62.15	0.53	0.47
treatment x sex *, [3]	3, 54.09	0.79	0.51
treatment x PCA1 *, [2]	3, 55.64	0.37	0.77
sex x PCA1 *, [4]	1, 60.89	0.67	0.41
treatment x sex x PCA1 *, [1]	3, 51.24	0.61	0.61
(d) Days PN22-36: body mass growth	d.f.	F	*p*
treatment	3, 60.85	2.88	0.14
sex	1, 64.94	5.23	0.02
PCA2	1, 69.55	18.28	< 0.0001
treatment x sex *, [3]	3, 54.31	1.13	0.34
treatment x PCA2 *, [4]	3, 60.23	1.74	0.17
sex x PCA2 *, [2]	1, 61.57	0.012	0.91
treatment x sex x PCA2 *, [1]	3, 52.31	1.56	0.21
(e)Day PN64: body mass	d.f.	F	*p*
treatment	3, 62.51	0.44	0.72
sex	1, 70.51	79.21	< 0.0001
PCA3	1, 69.58	57.30	< 0.0001
treatment x sex *, [3]	3, 61.12	0.68	0.57
treatment x PCA3 *, [2]	3, 62.69	0.47	0.70
sex x PCA3 *, [4]	1, 57.12	2.94	0.09
treatment x sex x PCA3 *, [1]	3, 57.38	0.12	0.95

3.4.2 Effects of pre- and post-hatching B exposure on the acute stress response

3.4.2.1 Day PN22

B stress response. As expected, B levels during the standardised restraint stress protocol were significantly affected by sampling interval (Table 3.3a). Overall, baselines were lower than both stress-induced B levels (post-hoc: T0 values *vs* T10 and T30 values: $p < 0.0001$ in both pair-wise comparisons), whereas there were no differences between B levels at T10 and T30 (post-hoc: $p = 0.49$). There were no effects of sex on B concentrations, nor any treatment effects (Table 3.3a). However, there was a significant interaction between treatment and sex in terms of the shape of the stress response (Table 3.3a). In fact, as shown in Figure 3.2a, in females, stress response patterns in the groups that experienced post-hatching B, regardless of pre-hatching experiences, peaked at T10 and then decreased between T10 and T30, while in groups that did not experience post-hatching B, stress levels peaked at T10 and tended, on average, to remain stable until T30. Juvenile males showed different patterns, with B levels peaking at T10 in all groups, apart from the BC males where stress levels tended to increase until T30 (Figure 3.2b). Post-hoc analysis confirmed that the post-hatching treatment was driving the observed sex-specific differences over the stress response in females (post-hatching B x sex x time interaction: $F_{4,101.66} = 2.79$, $p = 0.03$; $p > 0.54$ for all the other interactions). High variation in the BC males at T30 was due to one individual showing B levels of 26.46ng/ml. As this data point did not bias the statistical model ($-0.4 <$ model residuals < 0.4) and its removal from the analysis did not change the significant patterns in the statistical outcome, this value was kept in the final model.

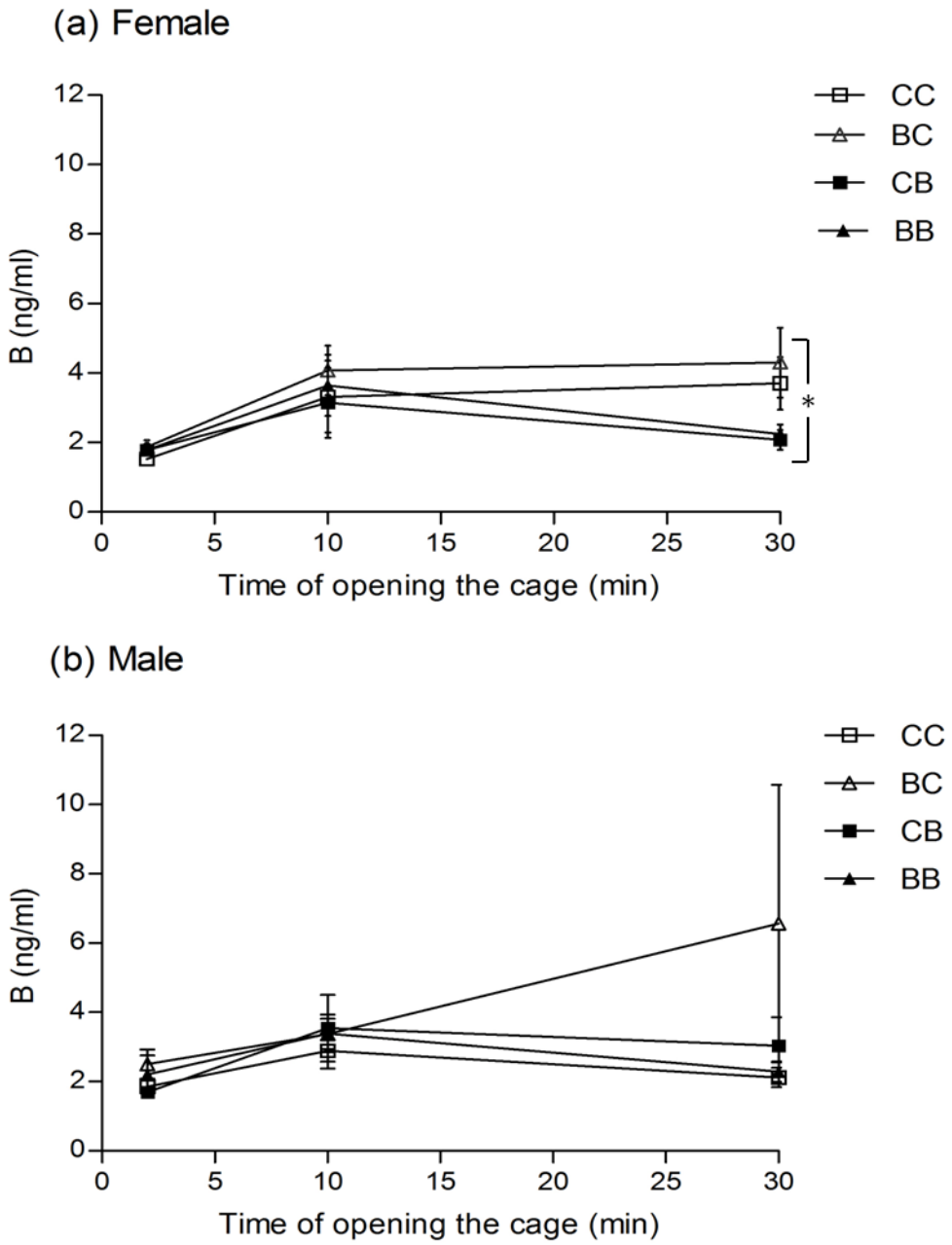

Figure 3.2.Corticosterone (B) temporal responses to acute stress (standardised capture-restraint stress protocol) across treatment groups (i.e. CC, BC, CB and BB) at post-hatching day 22 in (a) female quail and (b) male quail. As can be seen, CB and BB females exhibited shorter stress responses in comparisons with BC and CC females while no significant differences were observed among males (Linear Mixed Model: post-hatching B x sex x time of sampling interaction, $p = 0.03$; * indicates significant differences). Sample sizes: CC female = 9, male = 14; BC female = 10, male = 6; CB female = 10, male = 10; BB female = 9, male = 9. Data represent un-transformed means ± s.e.m. and included undetectable samples that were assigned individual detection limits.

Glucose, glycerol and triglyceride stress responses. Full descriptive statistics of basal and delta glucose, glycerol and triglyceride concentrations are reported in Table 3.4a. Basal glucose concentrations were not affected by treatment, sex or their interaction (Table 3.5a). In all juvenile quail, acute stress raised glucose concentrations, but such increases did not differ across treatments or between males and females (Table 3.5a). Basal glycerol concentrations were significantly higher in females than males regardless of the treatment group (females: 0.16 ± 0.01mg/ml; males: 0.12 ± 0.01mg/ml) and there was no effect of developmental B exposure on this response variable (Table 3.5a). Similar to the glucose response, overall glycerol levels tended to increase at the end of the stress protocol, but there was no effect of treatment, sex or their interaction (Table 3.5a). Regardless of treatment, juvenile females showed higher basal triglycerides than males (females: 1.49 ± 0.07mg/ml; males: 1.19 ± 0.10mg/ml). There was an effect of treatment on basal triglycerides, which was both sex- and glucose-dependent (Table 3.5a). Post-hoc analysis revealed that these complex interactions were driven by post-hatching B (pre-hatching B x sex x glucose interaction: $F_{1,\ 56.16} = 0.37$, $p = 0.55$; post-hatching B x sex x glucose interaction: $F_{1,\ 57.29} = 4.25$, $p = 0.04$; $p > 0.1$ for all the other interactions). In fact, the juvenile males, and not the juvenile females, that were treated post-hatching (i.e. CB and BB) tended to have lower basal triglyceride and higher glucose levels compared to the birds that were not treated with B post-hatching (i.e. CC and BC) (Figure 3.3b and Figure 3.4). Moreover, all the B-treated females showed on average higher basal triglycerides than the CC birds (Figure 3.3a).

In all the juvenile quail acute stress decreased triglycerides (Table 3.4a), but such a decrease did not differ among the treatment groups or sex (Table 3.5a).

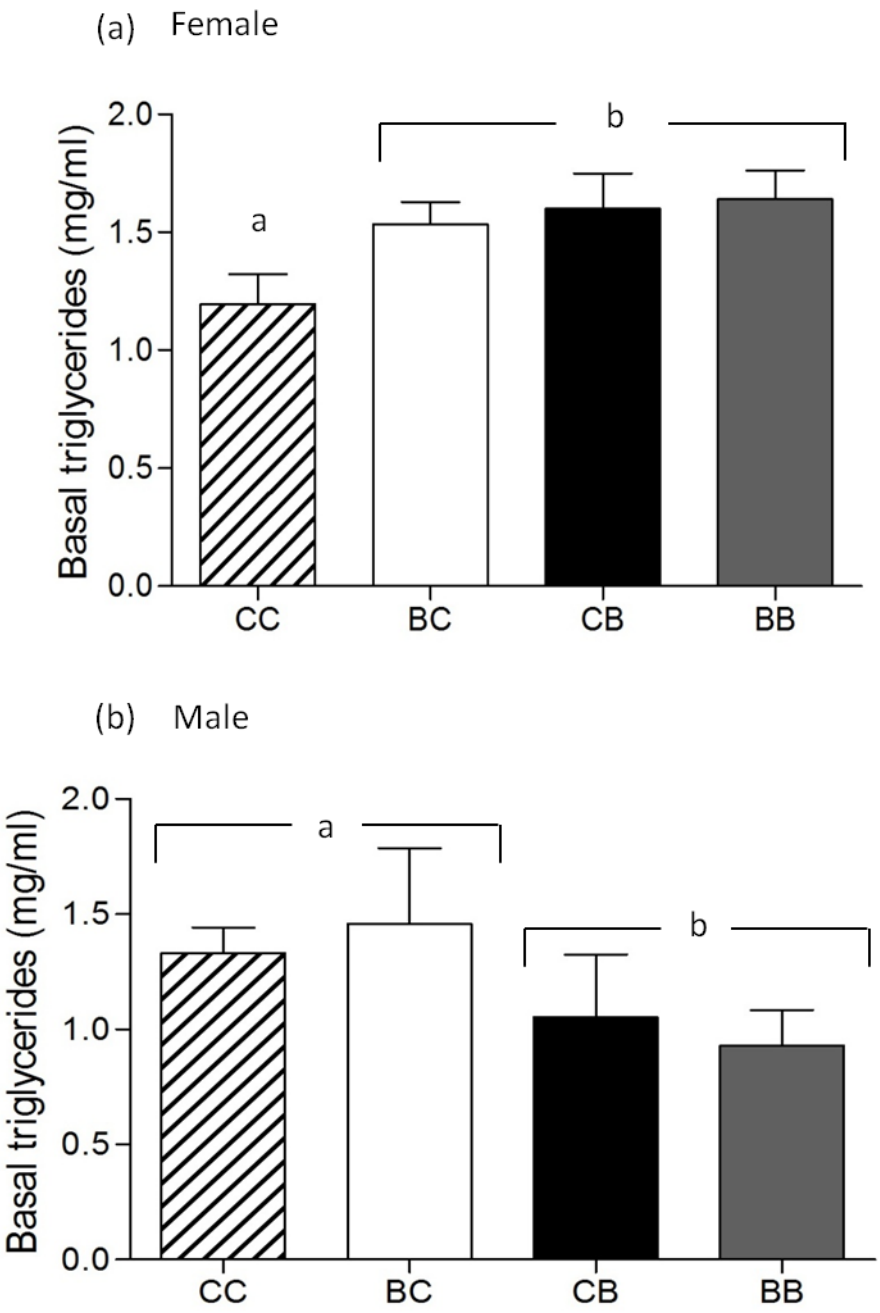

Figure 3.3 The interaction between corticosterone (B) exposure and sex in relation to basal triglyceride concentrations at post-hatching day 22 in (a) female quail and (b) male quail. Triglycerides were higher in all the B-treated females (BC, CB, BB) compared with the CC females; whereas males in the CB and BB groups showed lower triglycerides compared with the males in the CC and BC groups (Linear Mixed Models: treatment x sex interaction, p = 0.04, different letters indicate significant differences).

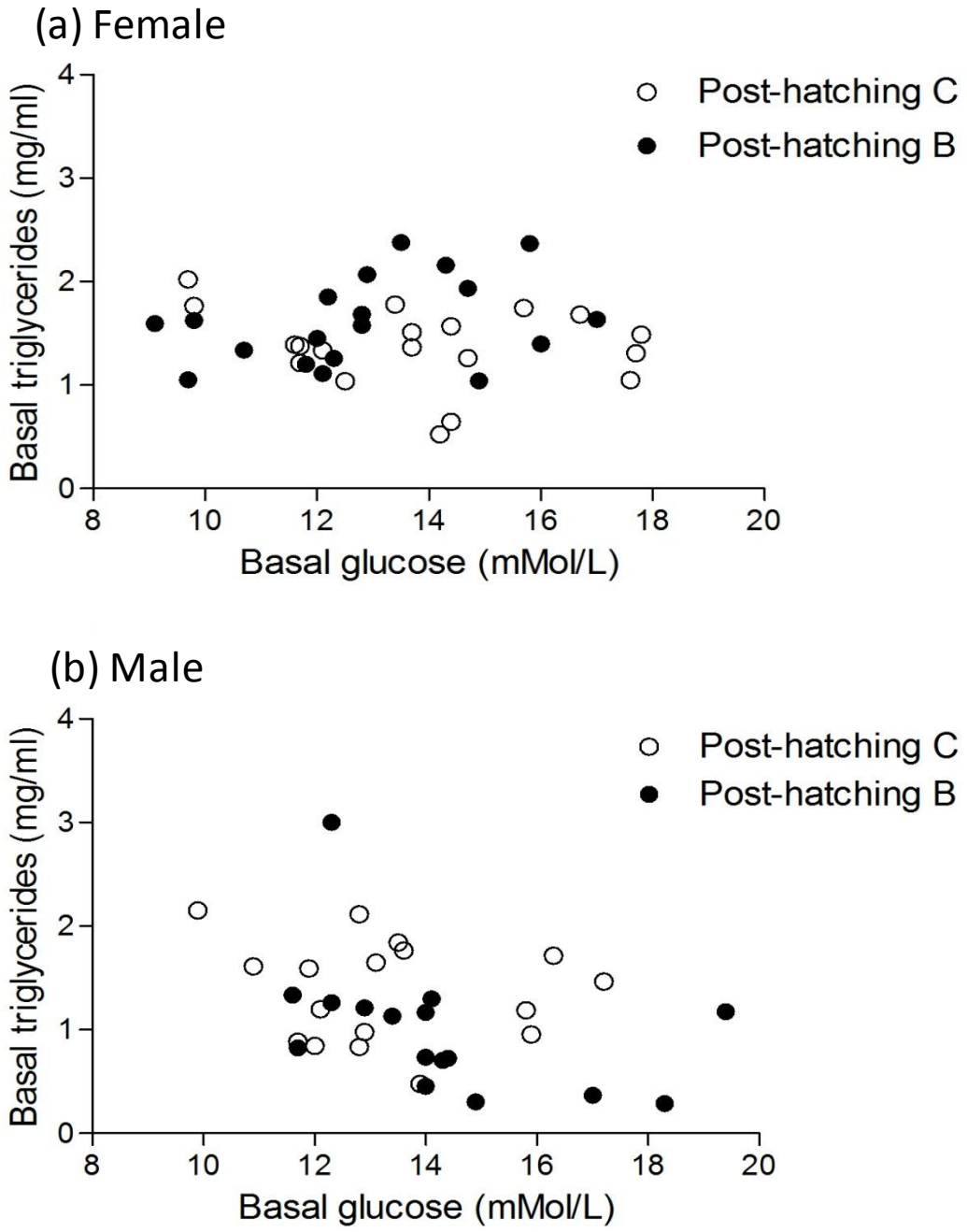

Figure 3.4 The correlation between basal triglyceride and basal glucose at post-hatching day 22 in (a) female quail and (b) male quail. As can be seen, in the males, and not in the females, triglyceride concentrations were explained by variation in basal glucose concentrations (Linear Mixed Models: post-hatching B x sex x glucose interaction, p = 0.04), with males exposed to B post-hatching (CB and BB) showing lower triglycerides and higher glucose concentrations and the post-hatching control males (CC and BC) showing opposite patterns. Sample sizes: CC female = 9, male = 12; BC female = 10, male = 5; CB female = 10, male = 9; BB female = 9, male = 7. Data represent un-transformed means ± s.e.m.

Table 3.3 Results of Linear Mixed Effect modelling (LMEs) of potential short- and long-term effects of treatment, sex and their interactions on HPA axis responsiveness at (a) post-hatching (PN) day 22 and (b) day PN64 (see text for details). Superscripts: * denotes excluded factors during the step-down procedure, numbers refers to the order of removal. In bold, significant factors ($p < 0.05$).

(a) Day PN22:	d.f.	F	*p*
treatment	3, 66.05	2.38	0.08
time	2, 99.71	27.09	< 0.0001
sex	1, 66.05	1.48	0.23
treatment x sex	3, 66.05	0.07	0.98
treatment x time	6, 99.71	0.49	0.82
treatment x sex x time	8, 99.71	2.38	0.02

(b) Day PN64:	d.f.	F	*p*
treatment	3, 70.79	3.25	0.03
time	2, 99.47	10.71	< 0.0001
sex $*,^{4}$	1, 69.02	3.17	0.08
treatment x sex $*,^{3}$	3, 64.09	0.24	0.87
treatment x time $*,^{2}$	6, 96.09	0.31	0.93
treatment x sex x time $*,^{1}$	8, 89.54	0.67	0.72

3.4.2.2 Day PN64

B stress response. Once again B concentrations during the capture-restraint protocol changed significantly over the stress response (post-hoc: T0 values *vs* T10 and T30 min values: $p < 0.0001$ in both pair-wise comparisons; T10 values *vs* T30 values: $p = 1.00$); however, there were no significant interactive effects (Table 3.3b; Figure 3.5). I found an overall effect of treatment (Table 3.3b) due to significantly higher hormone concentrations in BC birds compared with CC birds (post-hoc: $p = 0.03$; for all the other pair-wise comparisons: $p > 0.12$). Overall plasma B concentrations in females tended to be higher than B concentration in males regardless of the treatment groups, but these differences did not reach statistical significance (Table 3.3b)

Glucose, glycerol and triglyceride stress responses. Full descriptive statistics of basal glycerol and triglyceride concentrations over the stress response are shown in Table 3.4b. I found a sex-specific treatment effect on basal glucose concentrations, but no main effects of treatment and sex (Table 3.5b). Post-hoc analysis revealed that the significant interaction was driven by pre-hatching B (pre-hatching B x sex interaction: $F_{1, 62.43} = 10.70$, $p = 0.002$; post-hatching B x sex interaction: $F_{1, 67.95} = 0.07$, $p = 0.79$). In fact, males and females that experienced pre-hatching B exhibited reversed basal glucose patterns compared with males and females that were not exposed to elevated B pre-hatching B, with increased levels in BC and BB males compared with CC and CB males, and decreased levels in BC and BB females compared with CC and CB females (Figure 3.6). Also, the combined early B treatments tended to affect basal glucose levels (interaction: $F_{1, 65.56} = 3.70$, $p = 0.06$), with no differences between the sexes (pre-hatching B x post-hatching B x sex interaction: $F_{1, 67.82} = 0.25$, $p = 0.62$). Contrary to what was observed early in life, glucose concentrations remained on average stable between T0 and T30, with no significant differences across treatments and sexes (Table 3.5b). Both basal glycerol concentrations and stress-induced glycerol levels were not affected by the treatment, sex or their interactions (Table 3.5b). Basal and stress-induced triglycerides were higher in females than males (T0: females: 12.18 ± 1.63mg/ml, males: 1.33 ± 0.25mg/ml; delta: females: -4.21 ± 1.40mg/ml, males: 0.20 ± 0.39mg/ml), but there was no effect of treatment or its interaction with sex (Table 3.5b). As observed earlier in life, overall acute stress tended to increase glycerol and decrease triglyceride concentrations (Table 3.4b).

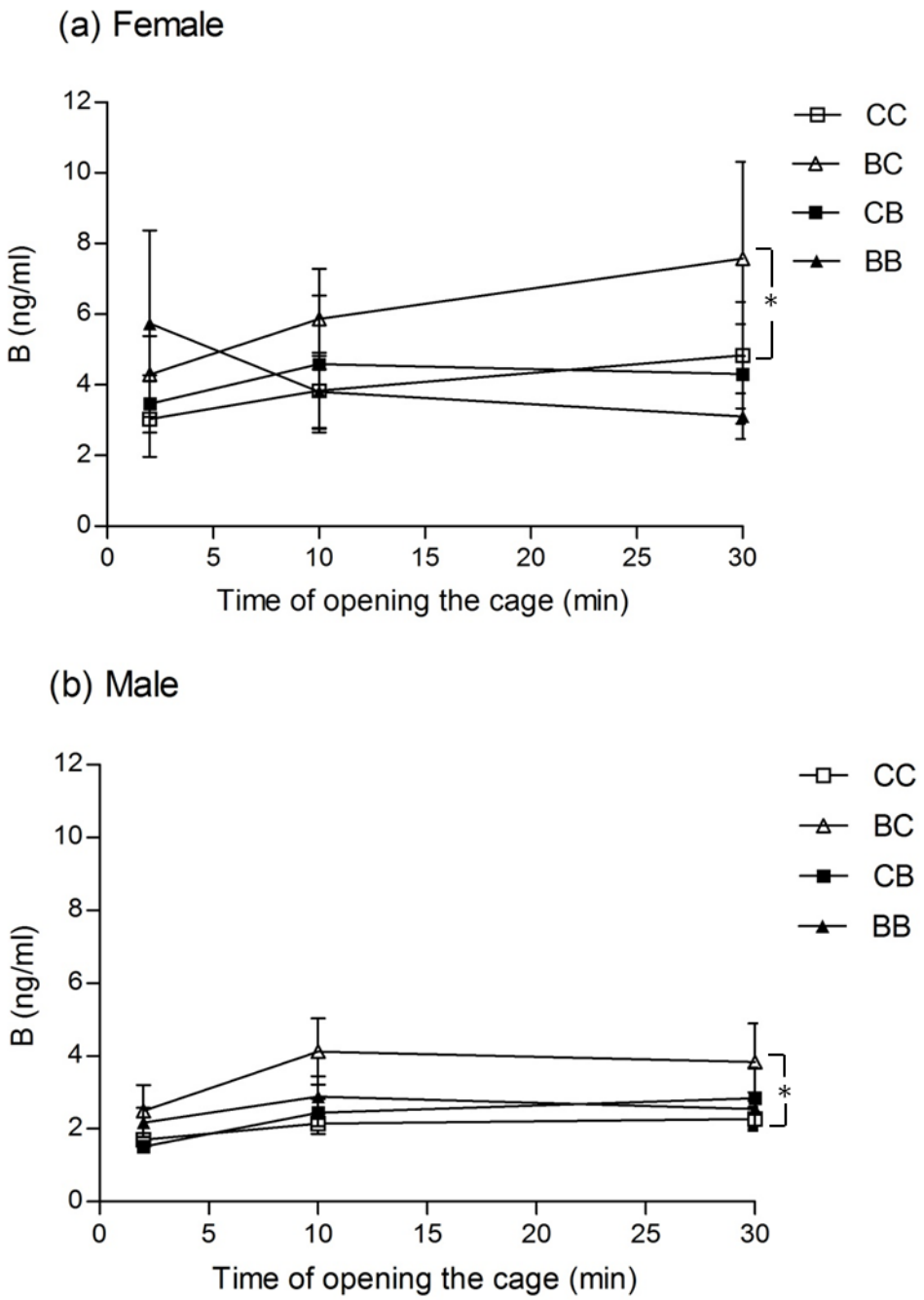

Figure 3.5. Corticosterone (B) temporal responses to acute stress (standardised capture-restraint protocol) across treatment groups (i.e. CC, BC, CB and BB) at post-hatching day 64 in (a) female quail and (b) male quail. Regardless of the sexes, hormone concentrations were overall higher in BC birds compared with CC birds (Linear Mixed Models: treatment, p = 0.03; * indicates significant differences). Sample sizes: CC female = 9, male = 14; BC female = 10, male = 6; CB female = 10, male = 10; BB female = 9, male = 9. Data represent un-transformed means ± s.e.m. and included undetectable samples that were assigned individual detection limits.

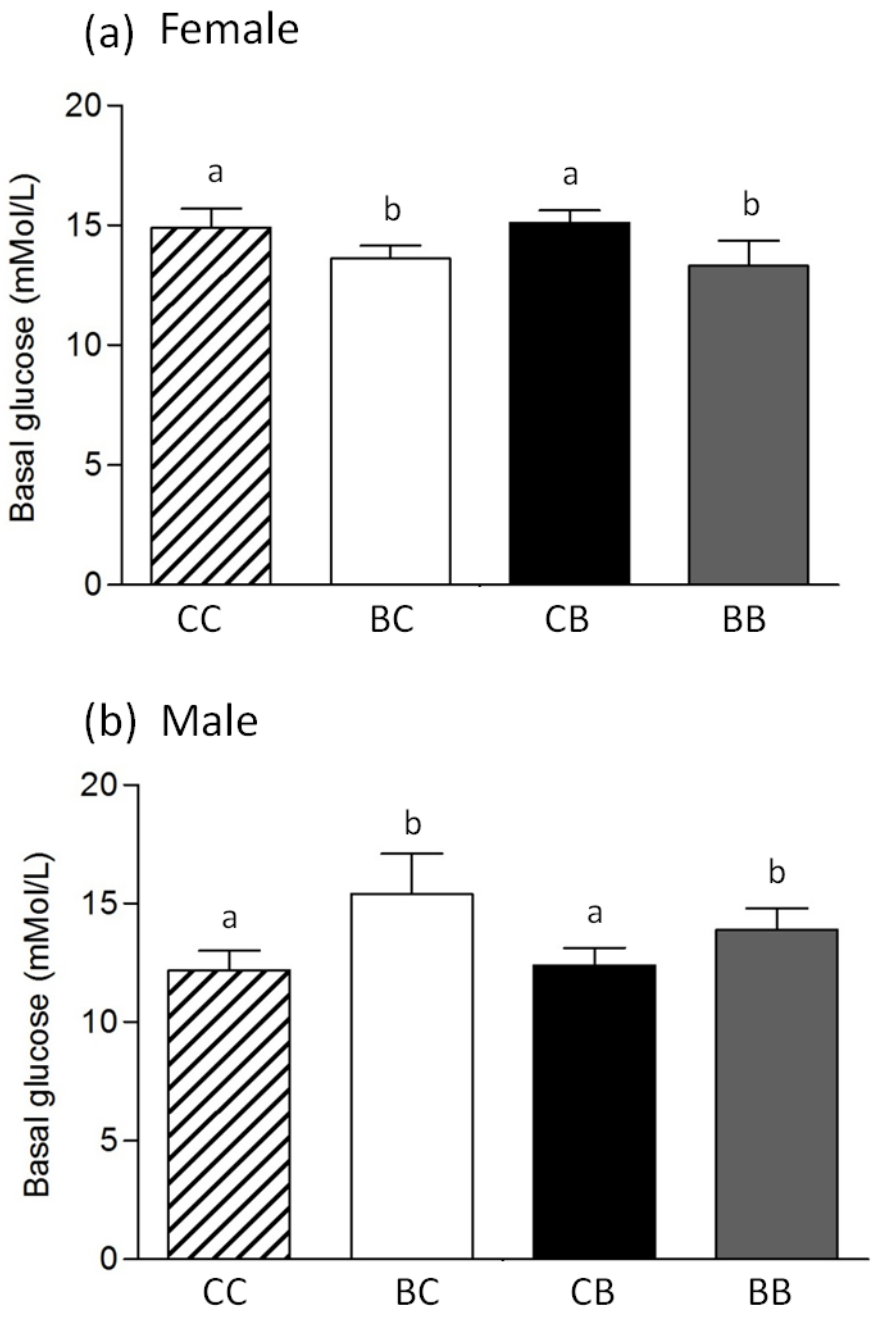

Figure 3.6. The interaction between corticosterone (B) exposure and sex in relation to basal glucose concentrations at post-hatching day 64 in (a) female quail and (b) male quail. As can be seen from the figure, glucose concentrations were lower in BC and BB females compared with CC and CB females, and higher in BC and BB males compared with CC and CB males (Linear Mixed Models, pre-hatching B x sex interaction, p = 0.002; different letters indicate significant differences). Sample sizes: CC female = 9, male = 14; BC female = 10, male = 6; CB female = 10, male = 10; BB female = 9, male = 9. Data represent un-transformed means ± s.e.m.

Table 3.4 Descriptive statistics of basal (T0) concentrations and acute stress responses (T30 – T0) of glucose, glycerol and triglyceride concentrations in (a) 22-day-old (day PN22) and (b) 64-day-old (day PN64) Japanese quail across the 4 treatment groups (CC, BC, CB and BB).

(a) day PN22

Glucose	CC		BC		CB		BB	
	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
T0	13.80	0.44	13.20	0.57	13.50	0.45	13.30	0.60
T30 - T0	1.60	0.41	1.00	0.36	2.40	0.53	1.20	0.43

Glycerol	CC		BC		CB		BB	
	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
T0	0.15	0.01	0.12	0.01	0.17	0.03	0.13	0.02
T30 - T0	0.09	0.04	0.07	0.02	-0.01	0.03	0.04	0.03

Triglycerides	CC		BC		CB		BB	
	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
T0	1.27	0.08	1.51	0.12	1.34	0.16	1.33	0.13
T30 - T0	-0.11	0.16	-0.54	0.12	-0.12	0.16	-0.29	0.12

(b) day PN64

Glucose	CC		BC		CB		BB	
	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
T0	13.26	0.62	14.26	0.74	13.73	0.53	13.59	0.68
T30 - T0	0.50	0.63	-1.00	1.02	-0.20	0.66	0.10	0.81

Glycerol	CC		BC		CB		BB	
	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
T0	0.13	0.01	0.22	0.05	0.24	0.05	0.18	0.02
T30 - T0	0.04	0.02	0.06	0.05	-0.02	0.04	0.09	0.03

Triglycerides	CC		BC		CB		BB	
	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
T0	4.66	1.56	9.07	2.89	8.40	2.11	6.37	2.15
T30 - T0	-2.31	1.28	-1.74	1.28	-2.84	2.19	-1.15	1.29

Table 3.5 Results of Generalised Mixed Effect modelling of potential short- and long-term effects of treatment, sex and their interaction on basal or delta glucose, glycerol and triglyceride concentrations (delta = difference between basal and stress-induced concentrations, see text for details) at (a) post-hatching day (PN) 22 and (b) day PN64. Superscripts: * denotes excluded factors during the step-down procedure, numbers refers to the order of removal. In bold, significant factors ($p < 0.05$).

(a) PN22			
Basal glucose	d.f.	F	*p*
treatment	3, 65.61	0.22	0.88
sex *,2	1, 68.94	0.07	0.93
treatment x sex *,1	3, 66.27	1.93	0.13
Delta glucose	d.f.	F	*p*
treatment	3, 66.14	1.26	0.29
sex *,2	1, 70.35	0.2	0.66
treatment x sex *,1	3, 63.05	1.77	0.16
Basal Glycerol	d.f.	F	*p*
treatment	3, 65.03	1.14	0.338
sex	1, 65.43	8.67	0.004
treatment x sex*1	3, 61.54	0.76	0.523
Delta glycerol	d.f.	F	*p*
treatment	3, 64.58	2.40	0.076
sex*2	1, 64.65	0.17	0.681
treatment x sex*1	3,60.47	0.60	0.614
Basal Triglyceride	d.f.	F	*p*
treatment	3, 53.32	2.85	0.046
sex	1, 54.81	8.17	0.006
treatment x sex	3, 52.14	2.92	0.042
Glucose	1,13.97	3.41	0.086
treatment x glucose	3,54.00	2.78	0.051
Sex x glucose	1,54.50	11.47	0.001
treatment x sex x glucose	3,51.98	3.71	0.017
Delta triglycerides	d.f.	F	*p*
treatment	6, 64.40	1.62	0.193
sex*2	1,64.60	2.10	0.152
treatment x sex*1	3,60.39	1.28	0.288

(b) PN64

Basal glucose	d.f.	F	*p*
treatment	3, 66.76	1.68	0.18
sex	1, 67.43	2.06	0.16
treatment x sex	3, 67.14	3.48	0.02
Delta glucose			
treatment	3, 60.21	0.45	0.72
sex *,[2]	1, 68.06	0	0.96
treatment x sex *,[1]	3, 60.95	0.65	0.58
Basal Glycerol	d.f.	F	*p*
treatment	3, 66.02	2.16	0.101
sex*[2]	1, 68.08	0.12	0.727
treatment x sex*[1]	3, 63.87	0.51	0.677
Delta glycerol	d.f.	F	*p*
treatment	3, 69.60	1.65	0.187
sex*[2]	1, 68.76	0.04	0.84
treatment x sex*[1]	3, 65.71	0.26	0.855
Basal Triglycerides	d.f.	F	*p*
treatment	3, 69	3.87	0.27
sex	1, 69	130.89	<0.0001
treatment x sex*[1]	3, 66	3.79	0.28
Delta triglycerides	d.f.	F	*p*
treatment	3, 69	2.83	0.42
sex	1, 69	5.32	0.02
treatment x sex*[1]	3, 66	0.45	0.93

3.5 Discussion

The present study clearly shows that the exposure to elevated stress hormones during development had an organisational impact on post-hatching HPA stress physiology. These findings are supported by previous studies and reinforce the hypothesis that the HPA activity is an important mediator to consider when addressing the effects of early life stress on shaping the phenotype. My experimental protocol involved a direct physiological manipulation of B exposure during pre- and post-hatching development. I am able, therefore, to attribute the effects induced by exogenous B to one (or both) of these developmental periods. To the best of my knowledge, this is the first avian study that attempted to quantify both the short- and long-term effects of developmental glucocorticoids on the HPA system, as well as glucose and lipid biochemistry during a standardised environmental stress test.

3.5.1 Pre- and post-hatching effects of B on HPA axis responsiveness

At day PN22, prolonged exposure to post-hatching B mediated HPA responsiveness in females but not in males, regardless of previous pre-hatching manipulations. Contrary to my predictions, therefore, elevated yolk B levels modified neither HPA axis function, nor the short-term effects of post-hatching treatment on the stress system. These results were unexpected as previous studies using similar manipulations in the egg, found significant effects on post-hatching growth, immunity or behaviours in juveniles of other bird species (Love *et al.*, 2005; Rubolini *et al.*, 2005; Saino *et al.*, 2005; Davis *et al.*, 2008). These studies were conducted in field conditions where a number of environmental confounding factors cannot be completely excluded. Also, variation seen across studies may also be explained by changes in HPA axis sensitivity across an individual's life cycle, especially during post-hatching growth (Schwabl, 1999; Sims and Holberton, 2002; Wada *et al.*, 2008; Chapter 2 of this thesis). Intriguingly, the effect of glucocorticoid exposure *in ovo* became evident only later in life. In fact, at day PN64, birds that had been exposed to B only during

their pre-hatching development (BC) experienced a higher total exposure to circulating concentrations of B during acute stress compared to the controls, indicating that HPA responsiveness in such treated birds was hyper-regulated. It should be noted that stress responses in the adult birds that experienced the combined treatments (BB) were similar to those observed in the control (CC) and post-hatching B-treated birds (CB). Taken together these results suggested that the experimental elevation of endogenous B post-hatching may have somehow "mitigated" the long-lasting impact produced by pre-hatching B exposure and reinforced the importance of interactive influences between these two time windows as shown in mammalian studies (Maccari *et al.,* 2005; Vallée *et al.,* 1997; Vallée *et al.,* 1999). As previously suggested, pre-hatching B may have modified HPA axis function through an elevation of basal circulating B levels (Coe *et al.,* 2003; Gutteling *et al.,* 2005). The undetectable samples, especially at T0, constrained my ability to test for potential treatment differences in baselines. Similar low B levels during restraint have been reported in the same species and are likely to be the result of frequent handling for morphological measurements (Hayward and Wingfield, 2004; Hayward *et al.,* 2006). In this study, I took morphological measurements in all the individuals at a specific age. The effect of handling, therefore, was standardised to all the birds. It is unlikely that the low hormone concentrations could be a sign of incomplete maturation of the HPA axis as birds from previous generations (same breeding population used in this experiment) were able to mount a B stress response at least from day 5 post-hatching.

There have been few studies in birds that explored the effects of pre-hatching glucocorticoid exposure on post-hatching HPA function, and the results of these are mixed. Studies during early post-hatching stages reported diminished HPA responsiveness in starlings at fledging (*Sturnus vulgaris*) (Love and Williams, 2008), and no effects or hyper-responsiveness in juvenile chickens (*Gallus gallus*) (Lay and Wilson, 2002; Haussmann *et al.,* 2012, respectively). Long-term studies in Japanese quail found HPA hyper-responsiveness in adults hatched from B-implanted mothers (Hayward and Wingfield, 2004) and, in contrast, HPA hypo-responsiveness in females, but not in males, when the hormone was injected directly in the yolk (Hayward *et al.,* 2006). This discrepancy has been explained by differences in the distribution of B in the egg when injected, as opposed to

when deposited by the mother (Hayward *et al.,* 2006). However, since the B dose used in this work raised yolk B levels to a similar physiological range as Hayward *et al.,* (2006), other factors may be involved. For instance, the timing of egg injection differed: in this study eggs were injected at day E5, whereas in Hayward *et al.,* (2006) injection was performed at day E0. In mammals the effects of pre-natal stress can change depending on the duration of early stress exposure (Kapoor and Matthews, 2008). Sensitive windows might also occur in birds and more comparative work is needed to investigate this hypothesis.

The sex-specific effect observed in this study following the post-hatching corticosteroid manipulation suggests that growing females are more susceptible than males to alterations in their stress responses after facing prolonged environmental perturbations. Studies in zebra finches (*Taeniopygia guttata*) suggest that females may be more sensitive than males to early post-hatching stress exhibiting lower growth patterns, reduced incubation effort and decreased survival (Verhulst *et al.,* 2006; Spencer *et al.,* 2010; although see Spencer and Verhulst, 2007). However, the short-term effects of post-hatching stress on HPA activity have been hardly explored. Previous work demonstrated that reduced food provisioning in nestling starlings induced an exaggerated peak in B release in response to acute stress at fledging, with smaller females showing the largest increase (Love and Williams, 2008). I measured the dynamics of the stress response, including the peak response, but also the change over time (between T10 and T30). My data suggest that in post-hatching B-treated females hormone concentrations returned to baseline more quickly than in post-hatching control females. This is indicative of changes in the duration, rather than the magnitude of adrenocortical B secretion. It has been proposed that post-natal/hatching stress may produce adaptive responses in the short-term, helping the juveniles to maximise their immediate chance of survival in low quality environments (Meaney, 2001; Love and Williams, 2008). Data from this study would support this idea as truncated stress responses could be an adaptive strategy to avoid the costs associated with high glucocorticoid concentrations (Wingfield, 2005b). Recent work in zebra finches showed that prolonged exogenous B in nestlings produced HPA hyper-responsiveness in adulthood and decreased survival (Spencer *et al.,* 2009; Monaghan *et al.,* 2012); similar HPA alterations were observed in maternally-deprived individuals into adulthood

(Banerjee *et al.*, 2012). This discrepancy reinforces the importance of considering the specific developmental strategy (precocial *vs.* altricial), as well as the life stage when investigating phenotypic effects of early life stress.

The mechanism underlying the short-term shift in HPA physiology observed in this study remains unresolved. Similar short-term alterations have been reported in rat pups subjected to daily handling during the first 21 days of post-natal life (Meaney and Aitken, 1985). Such changes have been linked to enhanced concentrations of glucocorticoid receptors (GR) in the hippocampus (Meaney and Aitken, 1985; see also Meaney, 2001 for a review), which are known to increase the efficiency of glucocorticoid negative-feedback (Sapolsky *et al.*, 2000). In fact, GR are predominantly occupied during acute stress when endogenous glucocorticoid levels are elevated; while mineralocorticoid receptors (MR) are largely occupied at basal concentrations of glucocorticoids and are thought to be primarily involved in feedback regulation during basal secretion (see General Introduction, Paragraph 1.2 and Figure 1.1 for more detail on the neuroendocrine regulation of the HPA axis). Therefore, modifications in the density of GR may explain the effects observed in this study in the post-hatching B-treated females. Although research on these systems in juvenile birds is lacking to date, recent work in adult birds showed that chronic stress can sensitise the HPA axis by altering central corticosteroid receptors (Hodgson *et al.*, 2007; Dickens *et al.*, 2009). More work is required to test the biological relevance of such factors in this model species.

3.5.2 Pre- and post-hatching effects of B in basal and stress-induced glucose, glycerol and triglycerides

Basal triglyceride concentrations were affected by developmental B in the juvenile 22-day-old quail. The treatment effect was inter-linked with the sex of the birds and basal glucose concentrations. Although these data are interesting, the interpretation of such patterns is not straightforward. First, it appeared that the B treatment, regardless of the developmental period it occurred, produced an increase of lipid biosynthesis in the juvenile females. As Japanese quail females are naturally heavier and fattier than the males, this may be indicative

of a higher susceptibility of females than males to short-term changes in lipid stores in response to early life stressful conditions. In the males, the effect of the treatment seemed more complex as it was dependent on post-hatching B treatment and basal glucose. The lower concentrations of triglycerides in the post-hatching B-treated males might be the result of enhanced available fuel via increase of glucose and free-fatty acids. This hypothesis would then fit well with the observed negative correlation between basal glucose and triglycerides among the post-hatching B exposed males and with the lack of these trends in the males that did not experience the post-hatching B protocol. In the awareness of the difficulties in disentangling causes *vs* consequences explaining these changes in the blood glucose and lipid chemistry, these data represent the first experimental suggestion in birds that prolonged exposure to developmental stress has the power to induce modifications in body energy balance pathways. Similarly, as seen with the B stress responses, later in life only the effects of pre-hatching B exposure persisted with changes in basal glucose, and again this effect was sex-linked. In fact, I found that the sexes were influenced by pre-hatching B exposure in opposite directions. The reversed patterns were more evident in birds treated only during their pre-hatching development. This metabolic alteration appeared in line with the long-term effect induced by pre-hatching B on adrenocortical activity and supports the hypothesis of links between basal B and basal glucose levels, as shown in other bird species (Remage-Healey and Romero, 2000). Furthermore, these data support findings in mammals where pre-natal stress has been shown to induce persistent changes in glucose metabolism (Vallée *et al.,* 1996; Nyirenda *et al.,* 1998; Lesage *et al.,* 2004; Benyshek *et al.,* 2006; although see D'mello and Lin, 2006), which can differ between the sexes (Franko *et al.,* 2010). There are indications that these alterations may compromise adult health and increase vulnerability to metabolic diseases (Cottrell and Seckl, 2009). I point out, however, that the nature of these data remains speculative as I still know little about how glucocorticoids influence energy balance and expenditure in birds.

I did not find any treatment differences in the responses to stress in any of the blood metabolites at any life stage. Other systems or hormones, such as the autonomic nervous system or insulin, may co-operate with the HPA axis in the transport and breakdown of energy in response to stress (Havel and Taborsky,

1989; Remage-Healey and Romero, 2001) and more studies are warrant in order to test the potential regulatory interactions. Finally, it should be noted that at day PN22 glucose increased at the end of the restraint in all the birds, regardless of the treatment. In contrast, triglyceride concentrations on average were diminished in response to stress, both in the juvenile and adult quail. These data support the hypothesis that lipids appear to be the primary source of energy in birds (Remage-Healey and Romero, 2001; Bairlein and Gwinner, 1994), but also raise the unexplored questions of different regulating mechanisms of the stress system in the stimulation of energy release depending on the individual's life stage.

3.5.3 Pre- and post-hatching effects of B on growth

Pre- and/or post-hatching B did not induce any significant effects on growth over the short- or long-term. This result was unexpected as stressful experiences often depress growth in the short-term (Eriksen, 2003; Hayward and Wingfield, 2004; Saino *et al.,* 2005; Janczak *et al.,* 2006; Spencer and Verhulst, 2007; Mueller *et al.,* 2009; Spencer *et al.,* 2009). Studies of pre-hatching stress in birds have also suggested that males are more affected than females (Love *et al.,* 2005; Hayward *et al.,* 2006; Love and Williams, 2008). Again, I did not find sex-specific patterns. This study adds to the few studies in birds that deviate from the above trends, finding no effects of developmental exposure to glucocorticoids on growth (Kitaysky *et al.,* 2003; Rubolini *et al.,* 2005; Haussmann *et al.,* 2012). In the long-term, the lack of treatment differences were expected as the growth decline in response to developmental glucocorticoid often disappear over the longer period (Hayward and Wingfield, 2004; Spencer and Verhulst, 2007; Spencer *et al.,* 2009). This can be obtained by a period of steady slow growth, or by a period of compensatory growth. The distinction is biologically important as compensatory growth can have long-lasting physiological costs (Metcalfe and Monaghan, 2001). I, therefore, conclude that the effects of pre- and post-hatching B treatments observed on the HPA axis physiology, triglyceride and glucose metabolism are direct effects of B itself and not indirect effects due altered growth trajectories.

3.6 Conclusion

In conclusion, the present study supports the hypothesis that developmental exposure to B can induce transient and permanent changes on the HPA axis and related metabolic pathways, which depend upon the developmental stage and sex. Importantly, I show that the impact of pre-hatching exposure to B on HPA axis functioning may be modulated by post-hatching stressful environmental conditions experienced during growth (here mimicked via oral administration of exogenous B). Although the underlying mechanisms of such shifts in the stress system are currently unknown in birds, these results corroborate findings in many mammalian models, suggesting that the organisational role of developmental glucocorticoid programming on the phenotype is a widespread phenomenon among vertebrates. Future longitudinal studies that can track potential dynamic changes of the effects of developmental stress into adulthood, from the peak of reproductive success until senescence, will be extremely useful to further our understanding of the long-term effects of developmental stress on fitness outcomes, survival expectancy and ageing processes. Furthermore, more studies are needed to link the physiological changes caused by developmental stressful environments with changes in stress-related behaviours and cognitive abilities in order to test predictions of the possible adaptive meaning of glucocorticoid programming. Finally, the results from this study emphasise the use of avian models in developmental research as they have the potential to tease apart indirect maternal and direct environmental stimuli acting on early life phenotypic plasticity. In fact, the high degree of variation in developmental strategies and life-histories in birds offer an excellent opportunity to undertake comparative approaches to further our understanding of potential ultimate costs and benefits of early life stress on young and aging phenotypes.

4. Chapter

Understanding the long-term effects of developmental stress in the hippocampus and hypothalamus: a functional genomic approach

4.1 Abstract

Developmental stress can potentially induce long-term phenotypic changes into adulthood. The sensitivity of developing individuals to stressful conditions can vary across differing developmental stages, producing a variety of phenotypes in later life. Although the mechanisms remain unclear, accumulating evidence suggests that such effects are mediated via programmed gene expression changes in specific brain regions primarily affected by the actions of glucocorticoid hormones. Here, using the Japanese quail as a study species, I examine the long-term effects of pre- and/or post-hatching exposure of the stress hormone corticosterone (B) on the hippocampal and hypothalamic transcriptomes in adulthood using RNA-sequencing technology. The two developmental hormonal manipulations result in four treatment groups: pre-hatching B exposed quail, post-hatching B exposed quail, pre- and post-hatching B exposed quail, and controls. Overall, the results suggest that the effects of developmental B on the brain transcriptome signature are strongly tissue-specific and the magnitude of these effects appears stronger in the hippocampus than in the hypothalamus. The analysis indentifies gene expression patterns that (1) respond in a similar way to both pre- and post-hatching B across the 3 B-exposed phenotypes relative to the control birds or, (2) respond specifically to one of the B treatment in the pre- or post-hatching B-treated birds relative to the pre- or post-hatching control birds. Furthermore, the dynamics of genes' responses are altered by the interactive effects of pre- and post-hatching B treatment, producing cumulative or compensatory effects in the birds that experience the combined B protocols. Taken together, these results highlight the importance of considering the effects of multiple stressors experienced

across the stages of development as they can give rise to differing adult phenotypes.

4.2 Introduction

An individual's phenotype is the result of its own genetic-make up and the environmental conditions that it has experienced over its life cycle (West-Eberhard, 2003; Monaghan, 2008). The integration of genetics in the field of behavioural and evolutionary biology is of central importance for the understanding of the genetic basis underlying phenotypic variation at both the population and individual levels (Jensen *et al.,* 2008). Until recently, this multidisciplinary effort was restricted to sub-systems of interest in order to identify candidate genes involved in the regulation of specific phenotypic pathways, such as those controlling metabolic activities or specific human diseases. However, there is an increasing appreciation that the interplay between the genotype and the resultant phenotypes is a complex phenomenon, often involving multiple inter-connected pathways (Agrawal, 2001; Pigliucci, 2005). Experimental investigations on a much bigger scale are, therefore, a more suitable tool in order to disentangle such complexity and examine overall genetic responses to changing environmental conditions (Rockman and Kruglyak, 2006).

The study of the long-lasting effects of early life experiences in later life has long intrigued human epidemiologists and biomedical scientists. It has been more than 30 years since the psychologist Robert Plomin started his research on twins, which is still ongoing. His studies have contributed to our understanding of the importance of the so termed "*non-shared environmental stimuli*" in shaping adult individuals with the same or very similar genetic starting material (e.g. Plomin *et al.,* 1977; Trouton *et al.,* 2002). Another fruitful area in this context is the earlier observational work in humans that has suggested a role of early life adversities, including small birth size and poor quality diet during neonatal life, in altering the susceptibility/propensity to adult diseases and mortality, generally known as "*developmental origins of health and disease*''

phenomenon (Barker *et al.*, 1990; recently reviewed by Gluckman *et al.*, 2007; Godfrey *et al.*, 2007). Despite several lines of evidence indicating that such links are likely to be very important, the underlying, and especially, causal mechanisms that impact on the nervous system, and consequently, on physiology and behaviour, are still relatively unexplored.

The Hypothalamic-Pituitary-Adrenal axis (HPA axis) is believed to be the main candidate mediator of the effects of early life stressful conditions on the phenotype. The mechanisms of actions of the HPA axis are highly conserved across vertebrates and have been described in detail in the General Introduction (Chapter 1, Section 1.2). Briefly, in response to a variety of stressors, hypothalamic corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) stimulate the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary into the circulatory blood system. ACTH is then carried to the adrenal glands where it stimulates the production and release of glucocorticoids. Glucocorticoids act primarily via two intracellular receptors: the lower affinity glucocorticoid (GR) and the higher affinity mineralocorticoid (MR) receptors and their mRNA expression and amounts in specific brain structures, especially in the hypothalamus and hippocampus, have a key role in terminating the stress response (Reul and de Kloet, 1985; de Kloet *et al.*, 1998). In mammalian models, the effects of circulating glucocorticoid concentrations in the hippocampus, a brain structure involved in learning and memory processes, are profound. Both studies *in vitro* and *in vivo* have demonstrated that such effects are mediated by MR and/or GR receptors via transcriptional activation or repression of target genes involved in a variety of cellular processes such as energy production; cellular metabolism; protein synthesis, trafficking and turnover; signal transduction; neuronal connectivity and excitability (Pearce and Yamamoto, 1993; Datson *et al.*, 2001). A shift in the balance of activated MR:GR ratio can alter such cellular processes with consequences for hippocampal circuitry integrity and synaptic transmission (de Kloet *et al.*, 1998). Additionally, chronically elevated concentrations of glucocorticoids are damaging for hippocampal neuronal viability, either directly or indirectly by increasing their vulnerability to neurotoxic and oxidative stress insults (McIntosh and Sapolsky, 1996; Sapolsky, 1996; Datson *et al.*, 2012). Although studies in different species than mammals are very limited, recent research in both domesticated and wild

birds indicate that the avian hippocampus is sensitive to the effects of both acute and chronic stress, with detectable changes at least in MR and possibly, in the MR:GR balance. In fact, adult zebra finches (*Taeniopygia guttata*) selected for exaggerated corticosterone (B) stress responses showed significantly lower hippocampal MR mRNA expression than non-selected adult controls, while no differences were detected in hippocampal GR mRNA expression between the two experimental groups (Hodgson *et al.*, 2007). Similar results were observed in chronically stressed adult European starlings (*Sturnus vulgaris*) within the hippocampus in comparison with non-chronically stressed adults (Dickens *et al.*, 2009). Since these changes in MR hippocampal gene expression density have been linked with changes in spatial cognitive performances (Hodgson *et al.*, 2007), it is likely that the effects of sustained elevated B levels have genome-wide effects in this brain area, similarly as in mammals. The effects of glucocorticoids in the hypothalamus have received much less attention than those induced in the hippocampus. However, there is line of experimental evidence *in vivo* demonstrating direct links between glucocorticoid supplementation and hypothalamic gene expression alterations in cell signalling and communication, metabolism and cytoskeleton regulation (Nishida *et al.*, 2006). To the best of my knowledge, no studies to date have examined simultaneously (within the same experimental individuals) the global gene expression pattern changes occurring in response to HPA axis activation/or its chronic stimulation across multiple target neuronal structures, such as the hippocampus and the hypothalamus.

The first studies that have analysed experimentally the effects of early life stressful events on the individuals' phenotype were carried out in laboratory rats. Such pioneering studies have demonstrated significant associations between naturally occurring maternal care variations and the development of stable individual differences in the adult offspring HPA stress responses. In fact, adult offspring of "high caring mothers" (i.e. high levels of licking, grooming and arched-back nursing behaviours) showed diminished HPA stress reactivity compared to the offspring of "low caring mothers" (i.e. low levels of licking, grooming and arched-back nursing behaviours) (Liu *et al.*, 1997). Intriguingly, cross-fostering studies have demonstrated that these physiological differences were reversed when the biological offspring of "low caring dams" were reared

by “high caring dams” (and vice-versa) (Francis *et al.*, 1999). These findings have raised the hypothesis that variations in maternal care could be an important non-genomic driver for programming the individual differences in the responses to stress. Follow-up research has indeed supported this hypothesis, revealing direct links between changes in the stress system and changes in the transcriptome (i.e. the complete set of transcripts in a cell/group of cells or tissue type/s and their quantity at a given time for a specific developmental stage or physiological condition) of the hippocampus in the adult offspring (Weaver *et al.*, 2006). These observed modifications involved less than 1% of transcripts of the total transcriptome (Weaver *et al.*, 2006); hence, the changes were subtle, but significant and involved transcripts regulating cellular metabolism and energy production; signal transduction and protein production, trafficking and turnover. Exposure to stress of gravid females can also have enduring effects on the offspring phenotypic trajectories, a phenomenon known as “*the foetal programming of the HPA axis*” (Seckl, 2001, 2004). Indeed, glucocorticoids of maternal origin do cross the placental and blood-brain barrier (Zarrow *et al.*, 1970). Maternal pre-natal stressors in rats can induce increases in the turnover of brain noradrenergic neurones in the adult offspring (Takahashi *et al.*, 1992); enhance the duration of stress-induced B secretion during acute stress as well as decrease hippocampal MR and GR receptors into adulthood (Maccari *et al.*, 1995; Barbanzanges *et al.*, 1996; Levitt *et al.*, 1996). Thus, both pre-natal hormones of maternal origin, likely glucocorticoids, and the effects of post-natal environmental stressors acting on the developing individual’s own stress system may be important modulators of the potential changes in the brain transcriptome controlling the HPA axis. Surprisingly, this hypothesis has never been experimentally tested.

Recently a number of experimental studies, carried out in models other than laboratory rats, have analysed the effects of early life stress on behaviour and physiology, and have highlighted the evolutionary inertia of developmental stress programming across vertebrate taxa (reviewed by Henriksen *et al.*, 2011; Love *et al.*, 2013). Much of this work has been conducted in bird species. Studies that have examined the long-term effects of direct pre- or post-hatching exposure to B have reinforced the idea that glucocorticoids can be key drivers in shaping phenotypic trajectories, including the response to acute stress (e.g.

Hayward and Wingfield, 2004; Hayward *et al.*, 2006; Spencer *et al.*, 2009; Marasco *et al.*, 2012 or Chapter 3 of this thesis). More recent avian literature has also emphasised the importance of manipulating both the pre- and post-hatching developmental environments in order to analyse experimentally the interactive actions of stressful events, which, indeed do occur and are likely to have biological relevance (Love and Williams, 2008; Marasco *et al.*, 2013 (Chapter 5); Zimmer *et al.*, 2013). To the best of my knowledge, the potential long-lasting effects of such interactions on the brain transcriptome profiles on target HPA axis structures have not been tested.

The recent development of high-throughput next generation transcriptome sequencing, also termed RNA sequencing (herein referred as "RNA-seq"), offers an attractive tool to map and quantify transcriptomes, and importantly, to link them with specific experimental conditions, for instance those defining a particular phenotypic conditioning (Wang *et al.*, 2009). In more detail, RNA-seq facilitates the conversion of a population of RNA (total or fractioned, such as poly (A) +) to a library of cDNA fragments and each molecule is then sequenced to obtain short-sequences (between 30-400 bases). These "short-reads" can be mapped onto the reference genome to produce a global transcriptome map, allowing gene expression quantification and differential expression analysis across differing biological conditions. RNA-seq provides several key advantages over other existing technologies, such as hybridisation-based approaches (i.e. tiling arrays, microarrays) or the more traditional Sanger sequencing platforms (i.e. cDNA- or Expressed-Sequence-Tag- sequencing) (reviewed by Wang *et al.*, 2009). For example, RNA-seq has higher sensitivity and a much larger dynamic range of expression levels over which transcripts can be detected when compared with microarrays (Mortazavi *et al.*, 2008); it also provides high levels of accuracy and technical reproducibility for the quantification of expression levels (Marioni *et al.*, 2008). Furthermore, RNA-seq does not necessarily rely on the genomic sequence of the study species as transcripts can also be assembled *de novo* without the use of the genomic sequence, although this approach of analysis is computationally more intensive and challenging (Oshlack *et al.*, 2010). However, it should also be noted that RNA-seq platforms and methods of analyses are demanding and still under active development. Some of the difficulties discussed in Wang *et al.* (2009) include the development of methods

to store, retrieve and process large amounts of data; aligning methods to reduce mapping errors, such as those resulting from sequence reads matching multiple locations in the genome; statistical methods to analyse complex experimental design, such as those with repeated measurements and more than two experimental conditions. While a lot of work has been done within the last three years to significantly improve alignment techniques (Trapnell *et al.*, 2012), the pace of progress regarding the statistical analysis is much less pronounced and more complicated than was originally expected (Auer and Doerge, 2010; Oshlack *et al.*, 2010).

The main aim of the present study was to examine the potential long-term effects of both pre- and/or post-hatching developmental stress on gene expression pattern of target neuronal HPA structures, the hippocampus and hypothalamus, using the Japanese quail as the study species. The present experiment was specifically designed to analyse to what extent the early post-hatching environment could directly alter the effects of previous pre-hatching maternal stress on hippocampal and hypothalamic gene expression patterns as well as to obtain an estimate of the tissue-specificity of such changes. According to the previously conducted work discussed above, I expected that both pre- and post-hatching exposure to stress hormones would induce changes in the hippocampus and hypothalamus of adult quail. I also expected that these changes might be modified in response to the combined interacting effects of pre- and post-hatching B as a result of enhanced induced-phenotypic plasticity in the developing quail (Monaghan, 2008). Finally since the effects of glucocorticoids are known to be tissue-specific, especially across differing brain structures (reviewed by Meijer *et al.*, 2003), I also predicted that the effects of the B protocols would differ depending on the brain region.

4.3 Methods

Experimental methods

4.3.1 Pre- and post-hatching exposure to B

The birds used in this study are part of the main experiment of my PhD project described in detail in Chapter 3 (Section 3.3.1). Briefly, pre-hatching stress was mimicked by exposure to corticosterone (B) *in ovo*; whilst post-hatching (PN) stress was mimicked via daily oral supplementation of B to the juvenile quail from PN5 to PN19. The combination of these two protocols resulted in four groups of experimental birds: (1) pre-hatching and post-hatching untreated birds (CC); (2) pre-hatching B-treated and post-hatching untreated birds (BC); (3) pre-hatching untreated and post-hatching B-treated birds (CB), and (4) pre-hatching B treated and post-hatching B treated birds (BB). Upon adulthood, PN days 69-73, the birds were sacrificed by intraperitoneal administration of 2ml of Euthatal (sodium pentobarbital, 200mg/ml; Merial Animal Health, Harlow, UK).

4.3.2 Isolation of hippocampi and hypothalami

The brains were removed within (mean ± s.e.m.) 10.57 ± 0.20 min *post-mortem*, immediately placed on dry ice and stored at -80ºC for further dissection. To perform the dissections, the brains were placed ventral side up into a frozen custom made brain holder (Workshop, University of Glasgow, UK) with a 1mm graduated scale, and a 2mm-thick coronal section was then obtained using two razor blades positioned approximately 4mm from the rostral pole and 2mm from the cerebellum. Two equivalent bilateral punches of the hippocampus (1mm diameter each) and one single punch of the hypothalamus (2mm diameter) were recovered from the slices using the brain topography of the chicken brain atlas as a reference (coronal brain sections interaural 2.08-2.56mm, Figures 18-20 in Puelles *et al.*, 2007; see also Figure A1 in the Appendix), such that tissue collection was standardised across animals. Tissues from hippocampus and hypothalamus were stored separately in collection tubes (Eppendorf, Hamburg, Germany) and placed back to -80 ºC until analysis. Hippocampi from 2 birds (1

BB group and 1 CB group) could not be obtained as they were damaged during the dissection process. The hippocampus and the hypothalamus were selected for the transcriptome analyses as both these brain areas have a key role in the regulation of the HPA axis. The high costs of the transcriptome analyses constrained the possibility to analyse other tissues, notably pituitary and adrenal glands, which are also fundamental HPA axis targets. Moreover, as the effects of elevated glucocorticoids during development can induce widespread effects in the nervous system at both the cellular and gene expression level (e.g. reviewed by Welberg and Seckl, 2001), the inclusion of another part of the brain to be used as a control (i.e. in which no gene expression changes would be expected) in the transcriptome analyses was not applicable in this experimental context.

4.3.3 RNA extractions

Total RNA was extracted using the Rneasy Microarray Tissue Mini Kit (Qiagen, Manchester, UK). Briefly, each tissue sample was homogenised in 1ml Qiazol lysis reagent. The homogenates were then spun for 5min (14000 x g) and the obtained supernatant was transferred to a new collection tube (Eppendorf AG, Hamburg, Germany). After addition of 200µl chloroform, the homogenate was shaken vigorously for 15s and incubated for 5min at room temperature. Homogenates were then spun (14000 x g) for 15 min at 4ºC to separate the aqueous and organic phases. One volume (500µl) of 70% ethanol was added to the upper aqueous phase, and transferred onto the Rneasy spin column, where the total RNA was bound to the membrane, while phenol and other contaminants were washed away. Then, a DNase digestion step was undertaken to remove potential DNA contaminants using RNase-Free DNase Set (Qiagen, Manchester, UK). After washing the Rneasy spin column membrane with 350µl of wash buffer (provided by the manufacturer), 80µl of DNase solution (DNase stock diluted 1:70 with the provided buffer) was added directly to the membrane and incubated for 15min at room temperature. The Rneasy spin column membrane was then washed, first with 350µl of wash buffer (the same as the one used before the DNase digestion step) and then with 500µl of a second type of wash buffer (both buffers were provided in the kit). The Rneasy spin column membrane was placed in a collection tube (Eppendorf, Hamburg, Germany) and RNA was then eluted in 30µl RNase-free water.

Both purity and integrity of RNA, two essential requirements for the overall success of RNA-based analyses (Bustin *et al.*, 2009), were assessed respectively using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Across all individual quail, hippocampal and hypothalamic RNA concentrations averaged (mean ± SEM) 158.86 ± 6.89ng/µl and 193.06 ± 6.39ng/µl, respectively. Agilent RNA integrity scores (RIN), which ranges from 1 (totally degraded) to 10 (intact), for the hippocampal and hypothalamic samples averaged (mean ± s.e.m.) 8.84 ± 0.06 and 9.16 ± 0.06, respectively.

4.3.4 RNA pooling

Total RNA extracted from the hippocampi and hypothalami of 48 randomly selected quail (out of 77 birds) were used for the transcriptome-sequencing. All samples conformed to the manufacturer's (Illumina, Little Chesterford, Essex, UK) quality requirements (RIN ≥ 8). For each tissue, 3 RNA biological replicates were prepared by pooling RNA from 4 birds (2 males and 2 females) per treatment, resulting in a total of 12 pooled samples per tissue. Each pool contained the same amount of RNA from each individual bird (500ng, 2000ng in total). The same pools of individuals were used for each tissue. Birds sharing the same mother were avoided within the same pool to control for potential pseudo-replication. The choice of pooling RNA samples was established *a priori*, when designing the experiment. In fact, RNA pooling is a common practice in transcriptome analyses due to their elevated costs and the approach of independently replicating biological pooled samples in each treatment condition is assumed to give a good estimate of the overall biological variability (Kendziorski *et al.*, 2003; Kerr, 2003; Rudolf *et al.*, 2013). I acknowledge that pooling males and females together may result in false negatives for the genes that are differently regulated between the two sexes. Despite the awareness of specific sex-specific differences in the effects of elevated glucocorticoid exposure during development on physiological and behavioural patterns (e.g. Henriksen *et al.*, 2011; Chapter 3 of this thesis), here, the approach of using both the sexes was preferred because the primary focus of the current study was

to gather global gene expression pattern differences across the treatments beyond the differences between the two sexes. Moreover, choosing only one sex would have reduced the number of birds in each biological pool, potentially increasing biological variation.

4.3.5 cDNA library preparation

The RNA pooled samples were then processed for RNA-seq using standard TruSeq™ RNA Sample Preparation kit (Illumina, Little Chesterford, Essex, UK). Briefly, the poly-A containing mRNA molecules were purified using poly-T oligo-attached magnetic beads. The mRNA was fragmented and the fragments were then synthesised into first strand c-DNA using reverse transcriptase. Then, second strand cDNA was obtained using DNA polymerase I and RNase H. In the next steps, cDNA fragments were processed in order to allow the ligation of the adapters at the fragment ends, generating flow-cell-suitable templates. The products were then purified and enriched by PCR to obtain the final cDNA libraries. Fragment distribution among the libraries was assessed using the Agilent 2100 Bioanalyzer DNA 100 chip. As indicated by the Manufacturer (see also Wang *et al.*, 2009 for a mini-review on cDNA library construction), fragment size averaged 309.16 ± 2.25 bases (mean ± s.e.m).

4.3.6 High-throughput sequencing

Sequencing was performed on a Genome Analyzer IIX (GAIIX) platform at the Glasgow Polyomics Facility (University of Glasgow, UK) following the Manufacturer's recommendations (Illumina, Little Chesterford, Essex, UK). The GAIIX relies on a flow cell with eight lanes (or channels) and massively parallel the sequencing of millions of short cDNA fragments in each lane. Briefly, the cDNA libraries were first hybridised onto the flow cell covered with complementary surface-bound primers. Each sample was loaded in one lane, resulting in a total of 24 lanes in 6 different flow cells; samples from different treatments were randomised across sequencing flow cells and lanes within flow

cells to control for sources of variation due to the flow cell and lane (Auer and Doerge, 2010). Hybridised cDNA templates were then extended via isothermal bridging amplification in order to create an ultra-high density sequencing flow-cell with approximately 30.000.000 clusters per lane. Each cluster contains about 1000 copies of the same template. The flow cell was then transferred from the Illumina cluster station to the genome analyser. At each cycle, the fragments in each cluster were sequenced using a four-color sequencing-by-synthesis technology that employs reversible terminators with removable florescent dyes (Sequencing reagents version 5, Illumina, Little Chesterford, Essex, UK) and the signals emitted recorded. The sequencing run terminated after 76 cycles and yielded reads (i.e. sequences of nucleotides) with a maximum length of 76 bases.

4.3.7 Microarray validation

4.3.7.1 RNA hybridisation

Microarray experiments were conducted at the Glasgow Polyomics Facility (University of Glasgow, UK). In order to perform a technical cross-platform comparison between RNA-seq and microarrays, Microarray libraries were constructed using the same total RNA hippocampal pools (n = 12, see Section 4.3.4. for details on sample preparation) that were previously used for the sequencing. Affymetrix GeneChip® Chicken Genome Array was used because: (1) quail and chicken are closely related species belonging to the same Family (Phasianidae); (2) genomic DNA hybridisation of quail to the chicken arrays showed that more than 80% of the signal probes were not statistically different between the two species, confirming high inter-specific DNA sequence conservation (Nakao *et al.*, 2008).

Prior the start of RNA hybridisation, the quality of all RNAs was re-assed by Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) to exclude potential degradation due to the length of storage (Figure A2, Appendix). As expected the obtained RIN numbers were all higher than 8 (range: 8.40-9.20),

conforming the Manufacturer's guidelines (Affymetrix Inc., Santa Clara, CA, USA). 500ng of total RNA per sample pool was used for library preparation using Ambion WT Expression kit, followed by Affymetrix GeneChip® WT Terminal labelling kit according to Affymetrix's instructions. Briefly, the workflow starts by generating first- and then second-strand cDNA, which are then synthesised in complementary RNA (cRNA) using *in vitro* transcription. Concentrations of cRNA in each sample were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA); cRNA quality was confirmed using the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). A total of 15µg of cRNA was used to synthesise sense-strand cDNA that was then processed for the fragmentation and terminal labelling. Prepared samples were hybridised for 16 hours at 45ºC on the GeneChip® Chicken Genome Arrays, which contained coverage of 32773 transcripts corresponding to over 28000 genes. The arrays were washed and stained using Affymetrix procedures on the Fluidics Station 450 and scanned on the GeneChip Scanner 3000 7G. Raw data from the scanned images (n = 12 files, CEL format) were generated using the GeneChip® Command Console Software (version 3.2, Affymetrix).

4.3.7.2 Genomic DNA hybridisation

In order to improve the sensitivity of the high-density oligonucleotide arrays when applied to closely related species I used the method developed by Hammond *et al*. (2005). This method allowed me to empirically determine the optimal hybridisation efficiency of the GeneChip® Chicken Genome Array probes when hybridised to the quail genomic DNA (gDNA), by masking out the probes which are inactive due to the difference in sequence between the species, as otherwise the hybridisation mismatches would attenuate the overall signal calculated across the probe-sets (Ji *et al.*, 2004). I extracted quail gDNA from approximately 6mg spleen tissue from 1 randomly selected individual (out of the 77 experimental quail) using the DNeasy Blood and Tissue kit (Qiagen, Manchester, UK). Then, I assessed concentrations and purity of the extracted gDNA using the Nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA). Then, a total of 500ng gDNA was labelled using the Bioprime DNA labelling System

(Invitrogen, Paislely, UK) and hybridised to the GeneChip® Chicken Genome Array, followed by washing, staining and scanning processes as described above. The GeneChip® Command Console Software (version 3.2, Affymetrix) was used to generate the raw data (n = 1 file, CEL format) of the scanned image.

4.3.8 Quantitative real time Polymerase Chain Reaction (qPCR) validation

Traditionally, qPCR is used to validate the gene expression levels measured by high-throughput technologies, such as RNA-seq and microarrays. Therefore, I compared the gene expression measures obtained by the above mentioned platforms with qPCR for the following 5 genes: vasotocin-neurophysin VT(the official gene symbol in the chicken genome is AVP as it is considered homologous to the mammalian gene encoding arginine vasopressin; however here herein referred as AVT to avoid misleading interpretations); transthyretin (TTR); superoxide dismutase extracellular 3 (SOD3); glutathione S-transferase Alpha 3 (GSTA3); guanine nucleotide binding protein (G-protein), Gamma 11 (GNG11) (Table 4.1). These genes were chosen for the technical validation across the three technologies for three main reasons. First, they are all biologically relevant: AVT is implicated in centrally regulated homeostatic processes and neuroendocrine responses to stress, as well as in the control of social and reproductive behaviours (reviewed by Goodson and Bass, 2001); TTR is a known carrier of thyroid hormones and retinal binding protein in the cerebrospinal fluid and its gene expression has been shown to be altered by maternal stress in rats (Kohda *et al.*, 2006; Wei *et al.*, 2012); SOD3 and GSTA3 are both encoding enzymes involved in cellular antioxidant defence processes; GNG11 plays a role in transmembrane signalling system and is thought to be involved in cellular senescence in humans (Hossain *et al.*, 2006). Second, the above mentioned genes were consistently differentially expressed in both the RNA-seq and microarrays experiments as measured with RankProducts analyses. Third, in both these technologies they showed maximal fold changes differences across the treatments. To minimise the technical source of variation, I used the same hippocampal RNA pools previously used for the RNA-seq and microarrays

analysis. All the qPCR analyses were carried out at the School of Psychology and Neuroscience (University of St. Andrews, UK) using a Stratagene MX 3005P (Agilent Technologies, Wokingham Berkshire, UK). Although RNA-seq analyses revealed other relevant genes to be differentially expressed (e.g. MR and BDNF, see Paragraphs 4.4.7.1 and 4.4.8.1), they were not selected for qPCR as they were not included in the top-gene lists and the main aim of qPCR was to achieve a technical rather than biological validation of the RNA-seq results.

Table 4.1 The 5 genes analysed by quantitative real time PCR (qPCR), cDNA sequence accession number from NCBI Reference Sequence Database (RefSeq: http://www.ncbi.nlm.nih.gov/refseq/) with forward (F) and reverse (R) primer sequence and probes (Perfect probe, FAM-labelled).

Gene	mRNA primer sequence 5'- to 3'	Accession number
Vasotocin-neurophysin VT (AVT)	F: ATGTGCCATGGACGCCG R: CCAGCACCGTCAGGTTCTT Probe: cTCCGCTGCCTCTTCTGCCTGCTCtcggag	NM_205185
Transthyretin (TTR)	F: ATGAATATGCTGATGTGGTGTTC R: GCAGTTGTTGAGTAAGAGAAAGG Probe: caACCGCCATTATACCATCGCTGCTCTCCTcggttg	NM_205335
Superoxide dismutase, extracellular 3 (SOD3)	F: CCAACCTCTTCGCCACAAT R: CAGCATTTCCATTTTCCAGACT Probe: acTTGCCCTTGCCCATGTCATCTTCCTGCgcaagt	XM_420760
Glutathione S-Transferase Alpha 3 (GSTA3)	F: CAGTTATTGAAGTTATGCCAAGATG R: GATTGTATTTCCCTGCGATGTAG Probe: aATCCCTGCTGTTCCATCAACTGCCACTGtgggatt	NM_204818
Guanine Nucleotide Binding Protein (G-Protein), Gamma 11 (GNG11)	F: GATGATCTGAGCGAGAAGGAC R: TCGGAGCACTTGGACACC Probe: TGCCTCTCCAGCTTCACTTCTTTCCGGAtgaggca	XM_00123433

4.3.8.1 Reverse Transcription

First-stranded cDNA was synthesised from total RNA (concentration: approximately 250ng) from each sample pool in a reaction mixture (50µl) containing Moloney-Murine Leukaemia Virus (M-MLV) Reverse Transcriptase

(200units/µl; Invitrogen, Life Technologies, Paisley, UK); random hexamers (50µM; Promega, Southampton, UK); dNTPs (10mM); Rnasin (40units/µl; Promega), dithiothreitol (DTT; 0.1M), and the appropriate volume of free DNAse/RNAse free water, similarly as described in Shaughnessy and Murphy (1993). The reaction was first incubated at 65ºC for 5min, then at 37ºC for 50min and finally at 70ºC for 15min. The cDNA formed was used as a template for qPCR.

4.3.8.2 Primer design and validation

Primers for amplifying the candidate genes were designed and validated by Primerdesign (Southampton, UK). The validation was conducted by testing the primers on a quail cDNA sample pool (prepared by pooling cDNA from 4 randomly chosen birds used in the present experiment) that I had provided to the Manufacturer. The specificity of the primers was regarded as acceptable when the following two conditions were achieved: (1) single sharp peak on the melting curve, and (2) good reproducibility between the expected thermocycling temperature for the amplification (Tm) and the observed Tm of the melting curve in accordance with the MIQE (Minimum Information for publication of qPCR Experiment) guidelines (Bustin *et al.*, 2009). The probes for each primer were then synthesised and tested again on the same provided cDNA template to assure the overall efficiency of the probe (Perfect probe, FAM-labelled). It should be point out that the gene AVT and the gene encoding mesotocin neurophysin MT have a distinct chromosome location in the chicken genome and the primers were designed in order to assure no cross-reactivity between these two genes.

4.3.8.3 Reference gene validation

The most common method to normalise qPCR data involves the use of a reference gene in order to control for variations in yield in RNA extraction, reverse-transcription and efficiency of amplification (Huggett *et al.*, 2005). A

fundamental characteristic of a reference gene is its invariant expression under the described experimental conditions. As recommended (Bustin *et al.*, 2009), I experimentally determined the optimal number and choice of the reference genes prior of conducting the qPCR assays (Vandesompele *et al.*, 2002). This study was carried out in a sub-set of cDNA hippocampal samples (n = 1 male and 1 female per each treatment group) randomly chosen among the experimental subjects used in this experiment. Briefly, gene expression levels of 6 candidate reference genes (1. ß-actin or ACTB; 2. Glyceraldehyde-3-phosphate dehydrogenase or GAPDH; 3. Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta Polypeptide or YWHAZ; 4. Succinate dehydrogenase complex, subunit A, flavoprotein or SDHA; 5. Splicing factor 3a, subunit 1 or SF3A1, and 6. Ubiquitin C or UBC) were determined using the geNorm kit (Primer design, Southampton, UK) by qPCR. For each qPCR reaction, 15µl of a PCR mixture and 5µl of cDNA sample (concentration: 5ng/µl) were loaded into the well of a 96-plate plate (Primerdesign, Southampton, UK). PCR mixtures were prepared daily separately for each reference gene following the Manufacturer's instructions so that each reaction contained: 1µl of primer provided in the kit (concentration: 300nM), 10µl of qPCR Mastermix containing SYBR green dye (Primerdesign *Precision* 2X qPCR Mastermix) and 4µl of RNAse/DNAse free water. All qPCR reactions were performed in duplicate in a unique plate; a reagent blank was included to detect potential contamination by genomic DNA. Thermal cycling conditions were: 10min at 95°C (enzyme activation), and 50 cycles of 15s at 95°C (denaturation step) and 1min at 60°C (primer annealing and elongation). The data were statistically analysed using the software "qbase" (Primerdesign, Southampton, UK). This software includes a module for geNorm analysis that calculates the gene expression stability measure (M) for a reference gene as the average pair-wise variation for that gene with all the other tested reference genes (Vandesompele *et al.*, 2002). Stepwise deletion of the gene with the highest M value allows ranking of the tested genes according to their M values. The analysis performed in the measured cDNA samples showed that YWHAZ and ß-actin had the lowest M values (0.30 and 0.32, respectively) relatively to the other candidate references ($M \geq 0.34$ for all the others). ß-actin was found to be the most stable gene in another independent experiment with similar pre- and post-hatching stressful

manipulations using brain tissues from adult quail that were obtained from the same local breeder (Cedric Zimmer's personal communication). Given my own results and the consistencies with the latter study, ß-actin was regarded as the most appropriate internal control in the present experiment.

4.3.8.4 qPCR assays

qPCR was performed using oligonucleotide primers and Taqman probes for ß-actin (reference gene), AVT, TTR, SOD3, GSTA3 and GNG11; sequences for all the primers and probes are reported in Table 4.1. qPCR were performed using the protocol Brilliant III Ultra-Fast qPCR Master Mix kit (Agilent Technologies, Santa Clara, USA). Each qPCR reaction mixtures (20µl) contained the provided Master Mix (10µl); gene-specific primers and probe (1µl); provided reference dye (diluted 1:500 in DNAse/RNAse free water) to compensate for non-PCR related variation in fluorescence; cDNA template (5µl; concentration: 1ng/µl), and finally DNAse/RNAse free water (3.7µl). Thermal cycling conditions were 3min at 95ºC (initial denaturation step) followed by 50 cycles of 15s at 95ºC and 20s at 60ºC (primer annealing and elongation). All qPCR reactions were performed in duplicate and a reagent blank (also in duplicate) per each gene was included within each plate. Gene expression measurements for the 6 genes from each individual bird were run in the same plate and individual birds from the different treatment groups were randomised across the plates (n = 14). The intra-plate coefficient of variation averaged (mean ± SEM) 1.02 ± 0.08%.

mRNA expression for all the genes was quantified using the comparative cycle threshold method (Schmittgen and Livak, 2008) relatively to ß-actin.

Data analysis methods

4.3.9 Raw data quality control

The reads obtained from the sequencing runs were quality checked using the Phred Quality (Q) scores (Ewing *et al.*, 1998). Q scores are defined as a property that is logarithmically related to the base calling error probabilities. For example, a Q score of 30 to a base is equivalent to the probability of an incorrect base call 1 in 1000 times (i.e. the base call accuracy is 99.9%). Therefore, Q scores ≥ 30 indicate high accuracy. Here, the raw sequencing data (containing both the sequences and the associated per base Q scores) were generated using Casava version 1.7.0 and stored in 24 files in fastQ format (Cock *et al.,* 2009). Initial standard investigations using FastQC software (version .10.0, http://www.bioinformatics.bbsrc.ac.uk) confirmed the presence of adapter sequences in small proportion (<0.5%) of 3'end of reads due to the corresponding cDNA fragments being shorter than 76 bases. It is standard procedure to remove the reads contaminated with the adapters to avoid problems with downstream analyses. I used an "*in-house*" software routinely employed in the Glasgow Polyomics Facility to remove these contaminated reads and re-analysed each individual sample using FastQC to ascertain the absence of adapter contamination (data not shown). I then used a Perl script written by Pawel Herzyk, which contrary to FastQC, allowed me to graphically represent the Q scores in each sequencing cycle per base-call and their associated standard deviations across multiple samples.

4.3.10 Trimming and mapping of the RNA-seq reads

Currently, there are two main valid strategies for assembling reads into genomic features, (1) "align-then-assemble" (alignment-first) or (2) "assemble-then-align" (assemble first or "*de novo* assembly") (Haas and Zody, 2010). In species lacking a reference genome (as with the Japanese quail), both approaches can use the genome of a closely related species for alignment purposes (e.g. Toth *et al.,* 2007). As the primary focus of the current project was to identify

potentially differentially expressed genes, without attempting to discover novel transcripts, the alignment-first approach was used. This approach confers the advantage of a predefined gene-space (i.e. the gene model of the related species), allowing for direct comparisons across treatments (Ward *et al.*, 2012). Remarkably, this method is also more sensitive for weakly expressed genes than the *de novo* assembly, which instead requires a relatively large sequencing depth across the transcript length (Francis *et al.*, 2013). The genome of the chicken (*Gallus gallus*) was chosen as a reference model for the following reasons: (1) both chicken and quail are closely related species and belong to the same Family (Phasianidae); (2) the high degree of conservation between the two species had been recently confirmed by comparative mapping of macrochromosomes (Kayang *et al.*, 2006) and by successful application of chicken Affymetrix microarrays to Japanese quail gene expression study (Nakao *et al.*, 2008); (3) the chicken model provides the best quantitative annotation in comparisons with the other avian sequenced genomes, the zebra finch and the turkey.

Preliminary alignment of quail reads to the chicken genome using the Bowtie aligning software (Langmead *et al.*, 2009) showed that only a very small percentage of reads (< 40%) aligned to the chicken reference genome when up to two mismatches were allowed. Consequently, the 76 bases long reads were shortened to 36 bases. The trimming of the reads also assured high quality reads (Q scores range: 38-34) with a flatter error profile along the read (Figure 4.4), and provided a reasonable compromise between the high number of aligned reads and the small number of reads mapped to more than one location in the genome (Pawel Herzyk's personal communication). The final read alignment was performed using TopHat version 1.3.2 (http://tophat.cbcb.umd.edu; Trapnell *et al.*, 2009), a transcriptomics read aligner able to align reads spanning the exonic borders. TopHat was provided with two source of information:

1. The genomic sequence of the chicken reference genome (FASTA file "galGal3" downloaded from http://genome.ucsc.edu and assembled by the International Chicken Genome Sequencing Consortium (2004), May 2006 release): this is a text-based format containing the nucleotide sequences of the reference genome in which the bases are represented using single-letter codes;

2. The chicken genome annotation (WASHUC2 release-65, GTF file Gallus_gallus.WASHUC2.65.gtf", downloaded from http://www.ensembl.org and appropriately modified to ensure chromosome name compatibility with the FASTA file): this file contains the localisation of the functional elements in the genomic sequence (e.g. protein coding genes and location of exons within a gene, promoters, tRNA and other RNAs).

Briefly, the TopHat pipeline first aligns the reads to the reference genome using the aligner Bowtie (Langmead *et al.*, 2009). Bowtie, however, does not allow alignments between a read and the genome to contain large gaps; hence, it fails to align the reads that span an exon boundary. TopHat pipeline can circumvent this limitation (Trapnell *et al.*, 2009; Trapnell *et al.*, 2012). First, TopHat collects the initially unmapped reads and then assembles the mapped reads. As explained in details in Li *et al.* (2008), during the assembly the mapped sequences flanking potential donor/acceptor splice sites (using the canonical GT...AG model as more than 99% of introns obey this rule; Mount, 1982) within neighbouring regions are joined together to form potential splice junctions. TopHat then breaks the initially unmapped reads into smaller segments and attempts to align each of them independently to the genome. The previously predicted splice junctions sequences are eventually confirmed when a certain number of a read's segments map to the genome far apart from one another (between 100 and several hundred kilobases).

For the final TopHat runs, the default parameters were used, except for a few parameters as detailed in Table A1 (Appendix). The deviations from the TopHat default settings allowed me to obtain a reasonably high number of quail aligned reads to the chicken reference minimising the change of reads mapped to more than one location in the genome (Pawel Herzyk's personal communication).

4.3.11 Quantification and normalisation of expression signal

Accurate quantification of the mapped RNA-seq reads requires the employment of normalisation methods in order to adjust for varying lane sequencing depths and potentially other technical and biological biases (Mortazavi *et al.*, 2008; Bullard *et al.*, 2010). Here, I used the TopHat output files (BAM format) to quantify and normalise RNA-seq reads using one of the following two methods of normalisation depending on the downstream type of analysis:

1. Number of Fragments mapped Per Kilobase of exon per Million reads mapped (herein referred as "FPKM") implemented in the software Cufflinks (Trapnell *et al.*, 2012);

2. Normalised read counts by combining the software HTSeq and Bayseq (Anders and Huber, 2010; Hardcastle and Kelly, 2010).

The FPKM measure of read density is a function of the molar concentration of a transcript in the starting sample by normalising for the transcript length and for the total read number in the measurement (Mortazavi *et al.*, 2008). This method, therefore, accounts for potential bias due to transcripts with different lengths within samples and between samples. In fact, longer genes are more likely to be detected as differentially expressed (Oshlack *et al.*, 2009). FPKM also controls for the potential overestimating expression values from the reads mapping to multiple position in the genome due to sequence repeats and homology (Mortazavi *et al.,* 2008). A full description of how FPKM were obtained using Cufflinks is provided in the Section 4.3.12.1 below.

The second normalisation approach does not take into account the gene length. Indeed, normalising the reads with respect to gene length was not crucial in this experiment as my objective was to compare the expression level of the same genes between samples and not to compare expression levels genes to genes (i.e. the biases will affect the same gene in the same way across samples). First, HTSeq (version 0.5.3p9; Anders and Huber, 2010; http://www-huber.embl.de/anders/HTSeq) was used to count how many reads map to each

gene. The software was provided with (1) the alignment files (SAM format; 1 file per sample; 24 files in total), and (2) the chicken annotation GTF file. SAMtools Perl Script was used to convert the TopHat alignment files from BAM to SAM format (http://samtools.sourceforge.net/; Li *et al.*, 2009). For the HTSeq run, the default parameters were used, except for the strandedness (this was set as "--stranded=no" because the reads from this experiment had not been made with a strand-specific protocol). The obtained raw counts were normalised in the software Bayseq prior to the statistical analysis (version 1.8.0, Hardcastle and Kelly, 2010; see also Section 4.3.12.2 below) using the 75th percentile of nonzero count distribution within each sample (Bullard *et al.*, 2010). This normalisation approach has been shown to be a more robust choice over the standard total-count normalisation, and the overall performance is best among several other existing normalisation methods (Bullard *et al.*, 2010).

4.3.12 Differential expression analysis

Due to the short-history of RNA-seq, the detection of differentially expressed genes is a challenging task. Currently there are still no standard procedures. Therefore, here, the data were analysed using three different statistical packages (Cufflinks, Bayseq, and RankProducts) and the results were compared. Cufflinks does expression quantification and normalisation itself employing the FPKM, which are then used to detect differentially expressed genes; whilst Bayseq and RankProducts use the normalised read counts.

4.3.12.1 Cufflinks

Cufflinks is an open-source package under continuous development and provides, together with TopHat and Bowtie, a complete RNA-seq workflow known informally as "tuxedo suite" (http://cufflinks.cbcb.umd.edu; Trapnell *et al.*, 2010; Trapnell *et al.*, 2012). Cufflinks provides two workflows, (a) "discovery mode" where transcripts are built up *de-novo* from the TopHat alignment data using Cufflinks and Cuffmerge modules, followed by differential expression

analysis within Cuffdiff module, or (b) "conservative mode" where differential expression is analysed using transcript definitions provided in the annotation GTF file only, and the Cuffdiff module is directly piped to the TopHat output. Here, I have applied the "conservative mode" as I was not interested in discovering new transcripts, and, preliminary testing of the "discovery mode" revealed a lack of consistency between different software versions (data now shown).

Cufflinks was provided with (1) the alignment TopHat files (BAM format; 3 biological replicates per treatment, 12 files in each run), and (2) the chicken annotation GTF file, appropriately modified to contain all the required attributes. With this approach, results from two different Cufflinks versions (1.3.0 and 2.0.1) were consistently similar at the gene expression level and, here, I reported the data from the most recent version at the time of the analyses (2.0.1, June 2012). As Cufflinks performs only pair-wise comparisons, I analysed treatment groups sequentially and separately per tissue, resulting in 6 contrasts per tissue (as shown in Table 4.4). Prior to statistical testing, Cufflinks fits a model of fragment count variances across replicates of each treatment. The variance is estimated using (1) the negative binomial distribution when a gene had a single isoform (Anders and Huber, 2010), or (2) the beta negative binomial distribution when a gene had multiple isoforms. For each gene, the $\log_2$-fold change between the FPKM values in two experimental conditions and their estimated variances produce a variable that is approximately normally distributed to which standard statistics can be applied (Student's t test, two-tailed); *p*-values are then adjusted for multiple testing using Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) and are reported as q-values (Storey, 2003). To enhance accuracy of differential analyses, the upper quartile normalisation ("--upper-quartile-norm") and the multi-mapped read correction ("--multi-read-correct") were enabled (Bullard *et al.*, 2010; Mortazavi *et al.*, 2008). The minimum number of alignments in a locus needed to conduct significance testing between samples was set to 1 ("--min-alignment-count", default is 10).

Exploratory Principal Component Analysis (PCA) plots were performed to assess sample grouping across replicates (Partek Genomic Suite, Partek Inc., St. Louis,

MO, USA), where samples were represented by an ordered sequence of transcript abundances.

4.3.12.2 Bayseq

Bayseq (version 1.8.0, Hardcastle and Kelly, 2010) is a package from the open-resource Bioconductor project (http://bioconductor.org; Gentleman *et al.*, 2004) implemented in the R environment (version 2.14.2; http://www.r-project.org). Similar to Cufflinks, Bayseq is a parametric statistical method that relies on the negative binomial distribution to estimate the variance within RNA-seq data (Hardcastle and Kelly, 2010). Differently from Cufflinks, however, Bayseq implements a Bayesian approach to empirically derive posterior probabilities (i.e. the conditional probability that is assigned after the relevant evidence, or axiom, is satisfied; Bolstad, 2007) of the observed data given a predefined model. Bayseq empirical modelling is based on the assumption that samples behaving similarly to each other should follow the same prior distribution, whereas samples behaving differently should have different distributions. Posterior probabilities are then converted to False Discovery Rate values (FDR).

Here, Bayseq was primarily chosen because it is the only package that enables analyses of experimental designs with more than two conditions. In a four condition experiment as in my study, there are 15 possible different model combinations: 1 in which there is no differential expression (NDE model) of any kind and 14 models showing differential expression (DE models). Of the latter there are 4 models in which 1 of the treatments shows differential expression compared to the other 3 treatments, 9 models in which 2 treatments show differential expression compared to the other 2 treatments, which can be combined together or considered as 2 independent groups, and, finally, 1 model in which data from all the 4 treatments are different from each other. All the models were performed separately in each tissue. Bayseq was also used to carry out pair-wise comparisons in each tissue, as described for Cufflinks.

4.3.12.3 RankProducts

RankProducts (Breitling *et al.*, 2004) is a well-established non-parametric method for the detection of differential gene expression in microarrays. RankProducts has been shown to be more accurate and powerful in comparisons with other classical approaches in the presence of noisy datasets with low numbers of replicates (Breitling and Herzyk, 2005; Jeffery *et al.*, 2006). Despite the large use of RankProducts in microarray experiments, this novel non-parametric method is not limited to microarrays analyses, but has the potential to be applied to a variety of "omics" (i.e. transcriptome, proteome and metabolome) studies as long as the data can be expressed as ranked lists (Breitling *et al.*, 2004). Briefly, RankProducts sorts experimental gene expression values by geometric mean of their ranks calculated over all pair-wise comparisons using all the sample replicates within a given pair of conditions. Ranks are calculated after sorting expression values by $\log_2$-fold changes within each contrast.

I analysed the data using RankProd package (version 2.28.0) available from the Bioconductor library (http://bioconductor.org; Gentleman *et al.*, 2004) implemented in the R environment (version 2.14.2; http://www.r-project.org). An important assumption of RankProducts is that the measurement variance is approximately equal across all the genes (Breitling *et al.*, 2004; Breitling and Herzyk, 2005), which can be met using the started-log data transformation (Rocke and Durbin, 2003). Here, a variant of the started-log procedure were used where: 4 different constants (1, 8, 16 or 32) were added to the normalised counts (produced by Bayseq) in each pair-wise comparison. The highest shifting factor (32) appeared to minimise the deviation from constant variance and was, therefore, chosen as the final normalisation algorithm [i.e. log2 (normalised counts + 32)] before performing RankProducts (Pawel Herzyk's personal communication). The data were analysed separately by tissue using a pair-wise approach as described in Cufflinks and Bayseq. RankProducts was carried out using the "data from single origin" option; ranks and *p*-values were calculated using 1000 permutations. The analysis controlled for the multiple testing error using the Percentage of False-Positives (PFP), which estimates FDR (Storey and Tibshirani, 2003).

4.3.12.4 Comparison among Cufflinks, Bayseq and RankProducts

The results obtained by Cufflinks, Bayseq and RankProducts were compared by analysing the genes in common across the three approaches using FDR ≤ 0.20 as cut-off. Less stringent cut-offs (0.30 < FDR < 0.20) have been shown to be a good strategy to minimise the loss of differentially expressed gene candidates when comparing statistical methods with different assumptions in the presence of noisy datasets (Zheng, 2012). The comparison was performed in each contrast (separately in hippocampus and hypothalamus) and separately in both the up- and down-regulated genes. I used the Ensembl identifier (http://www.ensembl.org) assigned per each individual gene to merge the datasets produced by the 3 packages using R (version 2.14.2; http://www.r-project.org). For the graphical representation of the comparative data I used proportional Venn diagrams (http://omics.pnl.gov/software/VennDiagramPlotter.php).

4.3.13 Vector Analysis

The results obtained from the RankProducts analysis were further processed within the Vector Analysis. Vector Analysis enables a comparison of gene expression changes across multiple experimental environments and a dynamic analysis of how individuals in each experimental environment respond to a specific common stimulus (Breitling *et al.*, 2005). Therefore, Vector Analysis was particularly appropriate in the context of this study because it allowed me to quantify the extent to which the expression of a given gene may have been modified in the adult quail by the independent or combined exposure to pre- and post-hatching B in the context of the different developmental environments. The basic principle of Vector Analysis is the transformation of expression changes of a given gene in two experimental environments into a unique vector (|Vsum|), which can be visualised in the Cartesian plane. The length of |Vsum| positively correlates with the consistency of the gene expression changes across all the possible pair-wise comparisons of the replicate samples; whereas the direction of Vsum indicates the type of behavioural prototype (Figure 4.1). Vector Analysis also assigns a non-parametric *p*-value to each prototype by

randomly sampling the measured expression values and calculating the |Vsum| for these random data. Therefore, by using pre-defined Vsum- and *p*- cut-off values, Vector Analysis confers a higher degree of objectivity than other existing graphical tools, such as Venn Diagrams, to further characterise the dynamics of genes' responses in multiple experimental environments.

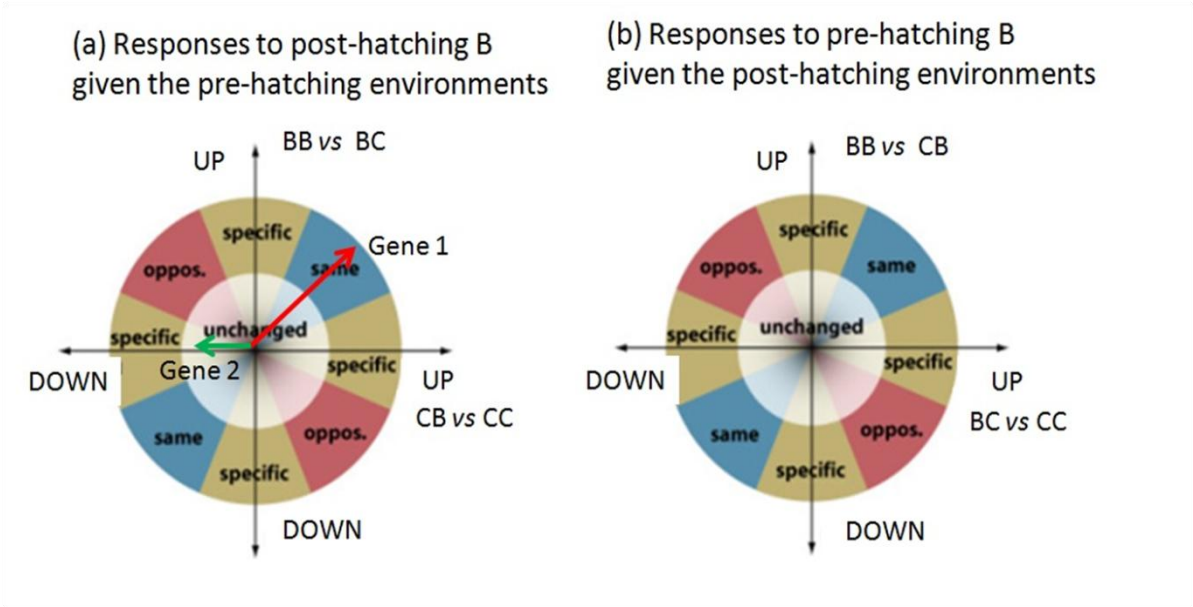

Figure 4.1 Graphical representation of the Vector Analysis performed to examine (a) the long-term responses of post-hatching corticosterone (B) exposure given the pre-hatching environments, i.e. control (horizontal axis) or exposure to B (vertical axis), and (b) the long-term responses of pre-hatching B given the post-hatching environments, i.e. control or exposure to B. On the two axes are reported the $\log_2$-fold changes of genes in response to the developmental environments. In (a) are reported two hypothetical vectors (|Vsum|): gene 1 (in red) is strongly up-regulated in both the environments; while gene 2 (in green) is specifically down-regulated in the control environment. In (a) and (b), the Cartesian plane is systematically sub-divided into sectors corresponding to the following prototypical behaviours: genes that show inconsistent responses in either environments ("unchanged", in white); genes that show similar responses in both environments (in blue); genes that show opposite responses in both environments (in red), and finally, genes that are specifically down-regulated in one environment and not in the other one (in yellow). From Breitling *et al.*, (2005) (*modif.*).

As Vector Analysis enables a two-dimensional representation at a time, here, I performed two separate analyses in each tissue using the genes that at least in one of the six contrasts showed FDR ≤ 0.10 in the RankProduct analyses (490 genes in the hippocampus and 302 genes in the hypothalamus). Then, I

compared these two analyses in pairs (Figure 4.1), a similar approach to that used in previous work (Kilian *et al.*, 2007). The first analysis focused on analysing the responses of post-hatching B given the pre-hatching environments, which were (1) "pre-hatching exposure to B" using the contrast BB *vs* BC (Figure 4.1a) or (2) "pre-hatching exposure to carrier only" using the contrast CB *vs* CC. The second analysis focused on analysing the responses of pre-hatching B given the post-hatching environments, which were (1) "post-hatching exposure to B" using the contrast BB vs CB or (2) "post-hatching exposure to carrier only" using the contrast BC vs CC (Figure 4.1b).

4.3.13.1 Behavioural categories

The data from these two analyses were filtered (using |Vsum| = 40 and p = 0.1 as cut-off values) in order to keep statistically significant and high consistency data. The resulted data were then decomposed into classes corresponding to the following behavioural categories:

I. ***Pre and post-hatching B responsive genes:*** the genes that showed similar significant responses in both the pre- and post-hatching environments as a consequence of exposure to B regardless of the developmental timing (Figure 4.2-I).

II. ***Specific pre-hatching B responsive genes:*** the genes that showed (1) no response to post-hatching B in either pre-hatching environments, and (2) similar significant responses to pre-hatching B in both the post-hatching environments (Figure 4.2-II).

III. ***Specific post-hatching B responsive genes:*** the genes that showed (1) similar significant responses to post-hatching B in both the pre-hatching environments, and (2) no response to pre-hatching treatment in either post-hatching environments (Figure 4.2-III);

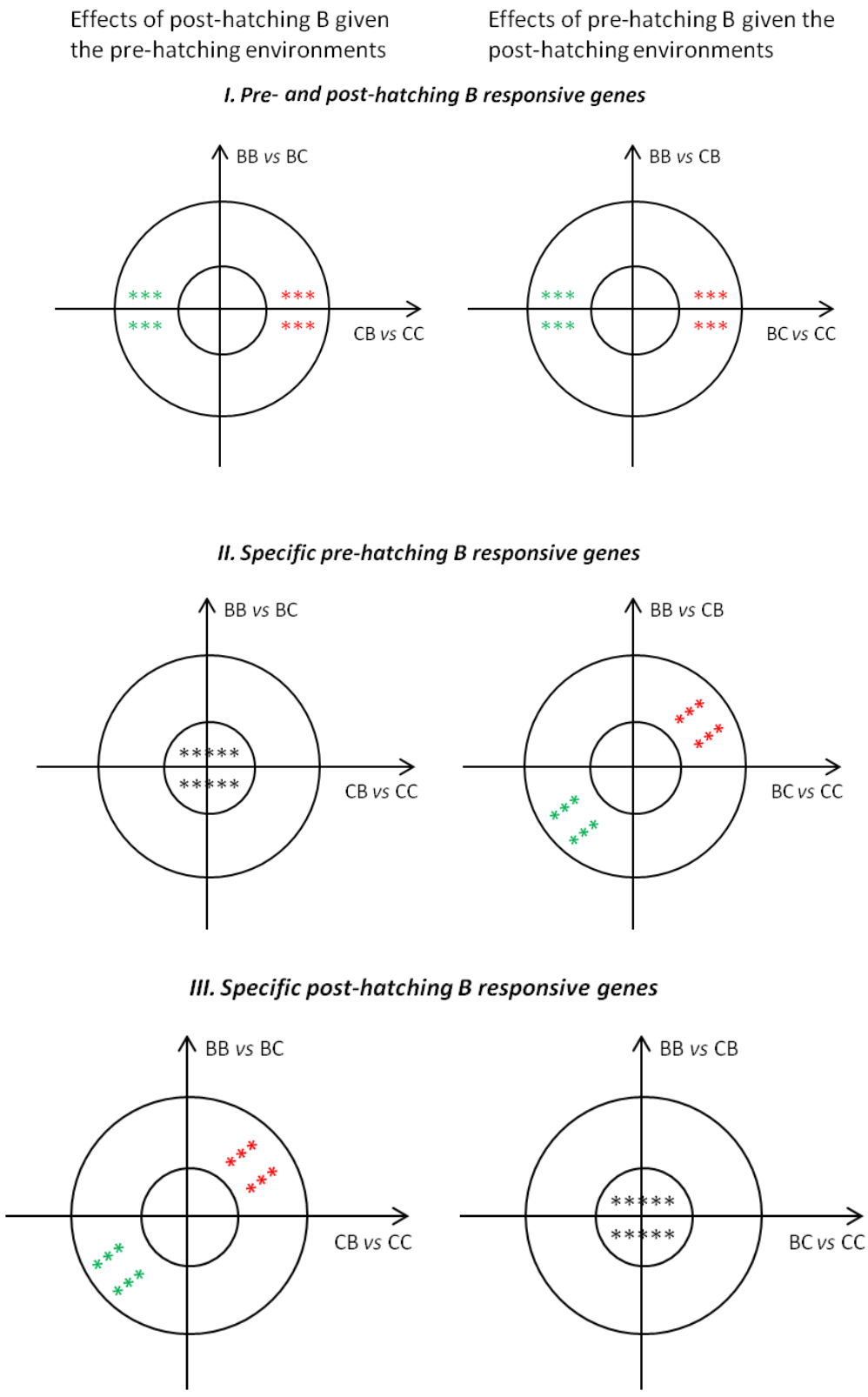

Figure 4.2. Graphical illustration of the behavioural categories (I, II, III) used to filter the Vector Analysis results. In all the graphs, the stars represent the vectors |Vsum| of hypothetical genes: coloured in red the significant up-regulated genes in one or both the pre- and post-hatching environments; in green the significant down-regulated genes in one or both the environments; and in black the genes whose responses were not significant specifically in one environment. A gene was significant when: |Vsum| ≥ 40 and $p \leq 0.1$ and non-significant when: |Vsum| ≤ 40 and $p \geq 0.1$.

Interacting pre- and post-hatching B responsive genes: the genes that showed (1) similar significant responses to post-hatching B in both the pre-hatching environments and (2) similar significant responses to pre-hatching B in both the post-hatching environments. In this category two distinct biologically relevant patterns were distinguished depending on whether the similar responses within the two environments are similar or opposite when compared between the environments. Specifically:

Similar between-environment responses:

IV. This implied a **"*cumulative effect*"** via elevating or attenuating expression depending on whether the fold changes BB/CB, BC/CC, BB/BC and CB/CC were all positive and similar, or, all negative and similar (Figure 4.3-IV).

Opposite between-environment responses: this implied a **"*null effect*", ** which could occur in two distinct ways:

V. Via elevating gene expression in the BC birds with the fold changes BB/CB and BC/CC both positive and similar to each other whilst the fold-changes BB/BC and CB/CC were both negative and similar to each other (Figure 4.3-V), or,

VI. Via elevating gene expression in the CB birds with the fold changes BB/CB and BC/CC both negative and similar to each other, whilst the fold-changes BB/BC and CB/CC were positive and similar to each other (Figure 4.3-VI).

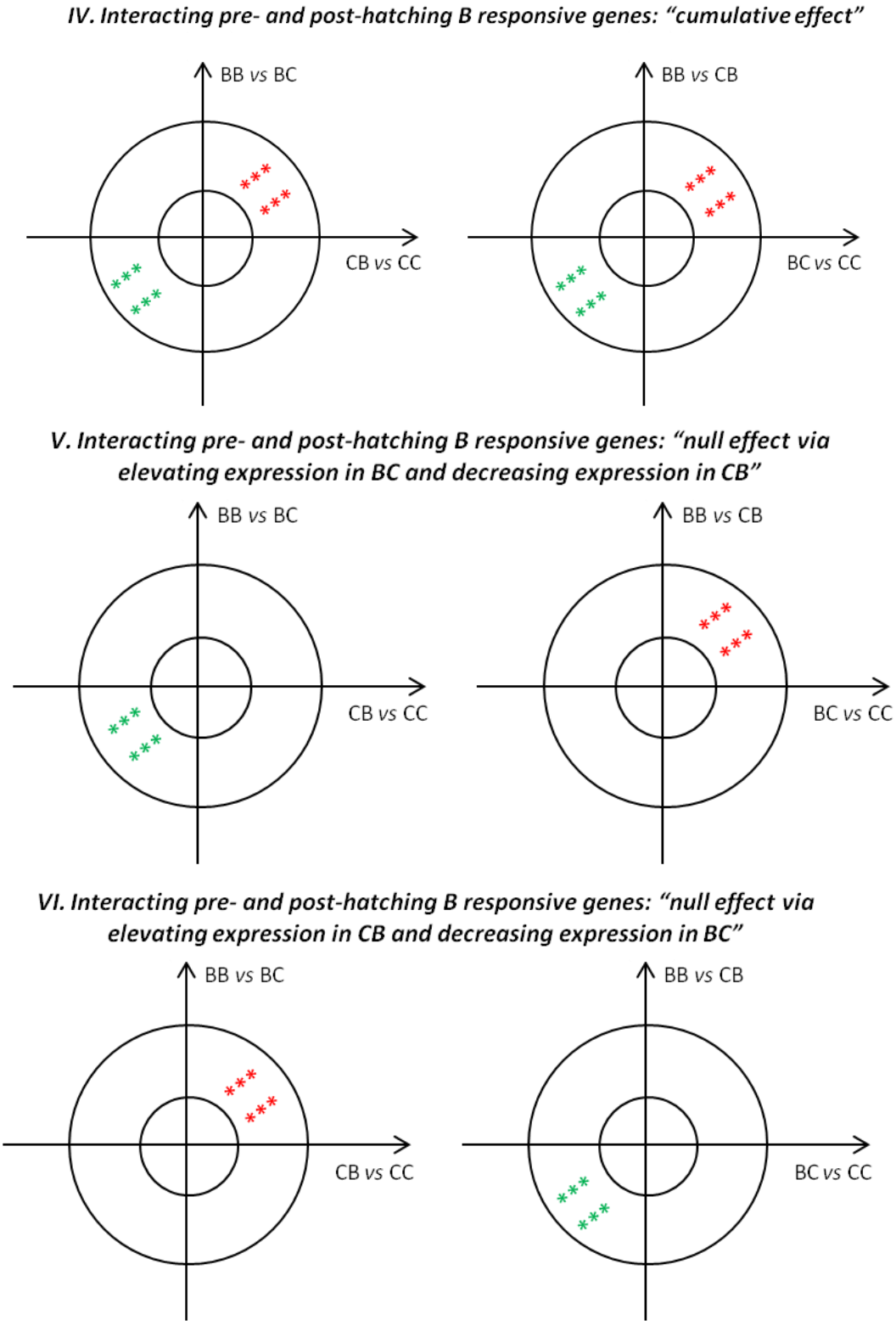

Figure 4.3. Graphical illustration of the behavioural categories (IV, V, VI) used to filter the Vector Analysis results. In all the graphs, the stars represent the vectors |Vsum| of hypothetical genes: coloured in red the significant up-regulated genes in one or both the pre- and post-hatching environments; in green the significant down-regulated genes in one or both the environments; and in black the genes whose responses were not significant specifically in one environment. A gene was significant when: |Vsum| ≥ 40 and $p \leq 0.1$ and non-significant when: |Vsum| ≤ 40 and $p \geq 0.1$.

4.3.14 Gene annotation and functional analysis

The web-based functional annotation tool "Database for Annotation, Visualization and Integrated Discovery" or "DAVID" (version v6.7, http://david.abcc.ncifcrf.gov/) was used to annotate the significant candidate genes identified by RankProducts and Vector Analysis. A unique list of Ensembl identifiers were uploaded via the web interface and the background was selected as *Gallus Gallus*.

Ingenuity Pathway Analysis (IPA, Ingenuity Systems, http://www.ingenuity.com/products/ipa) was used to identify possible biological processes associated with the differentially expressed significant gene lists obtained after applying the behavioural categories as described above (Section 4.3.13.1). For each gene list containing at least 14 genes, a unique list of gene identifiers (Ensembl IDs) was submitted to the IPA server. Here, each Ensembl IDs was mapped to its corresponding gene object in the Ingenuity Knowledge Base (IKB) via ortholog mapping (to their vertebrate counterparts including Human, Mouse and Rat). All information in the IKB is curated from the published literature and co-functioning genes are supported by evidence extracted from the underlying publications. The IKB converts each submitted gene list into a shorter dataset of well-characterised, non-redundant "focus" genes. These genes are then projected onto a global molecular network generated from the information contained in the IKB and a number of small networks (here, up to 35 genes in total per network, default parameter) can be algorithmically generated on the basis of their inter-connectivity. Subsequently, for each network the genes associated with biological functions are identified and Fisher's exact test is then used to calculate *p*-values, determining the probability that each biological function assigned to a given network is due to chance alone. The Fisher exact test *p*-values are converted to the score equal to $-\log_{10}(p\text{-value})$. The whole dataset of the "focus" genes is then analysed in order to identify the most representative gene functional and canonical pathways classes. The "focus" genes associated with the canonical pathways are identified and the significance of such association is measured in two ways:

1. A ratio of the number of genes from the datasets that map to a given pathway, divided by the total number of genes that map to that pathway, is displayed;

2. The Fisher's exact test is used to calculate *p*-values and determines the probability that the association between the genes in the dataset and a given canonical pathway is explained by chance alone.

Finally, IPA analysis also identifies the "upstream regulators" (i.e. any molecule, such as a transcription factor, cytokines, receptors or other chemicals, that can affect the expression of another molecule) that may be responsible for the observed gene expression changes in order to enhance the understanding of the biological activities occurring in the analysed tissue/s or cell/s.

4.3.15 Microarray data analysis

4.3.15.1 Probe selection using gDNA hybridisation

The raw data (n = 1 file, CEL format) obtained from the hybridisation of quail gDNA to the GeneChip® Chicken Array contained hybridisation intensities between the Japanese quail genomic fragments and all the chicken probes. The GeneChip® Chicken Array use probe-sets, each comprising 11 probe-pairs to quantify abundance for each transcript. Each probe-pair consists of a perfect-match (PM) and a mismatch (MM) probe: the PM probe is a 25-base sequence complementary to the target transcript, while the MM probe is identical to the PM probe except for the presence of a single mismatch at the 13th base. The information about positioning of individual probes on the array and grouping the probe-pairs into probe-sets is specified in the chip definition file (cdf file). Using Xspecies software (version 2.1, Hammond *et al.,* 2005) I have modified the GeneChip® Chicken Array cdf file so that it would retain only the probe-pairs in which the PM probe has a gDNA hybridisation intensity signal greater than the user predefined threshold (Hammond *et al.,* 2005).

As I did not have any *a priori* definition for threshold intensity settings, I performed a pilot study (i.e. Hammond *et* al., 2005; Graham *et al.,* 2010) to determine the best intensity value that could maximise the removal of probe-pairs that hybridized weakly, but, at the same time, minimising the loss of probe-sets. The new modified cdf files were produced with the following gDNA hybridisation threshold intensities: 50; 100; 150; 200; 250; 300; 350; 400; 450; 500; 600; 700; 800; 900 and 1000. Consistent with the previous studies (Hammond *et* al., 2005; Graham *et al.,* 2010), Figure 4.4a shows that the number of probe-pairs retained in the probe mask files reduced rapidly at increasing thresholds, while the number of probesets retained reduced at a much slower rate. At gDNA hybridisation intensity threshold of 200, 62.24 % of probe-pairs were removed in the probe-mask file, whilst 95.19 % of probesets were maintained. As at threshold intensities higher than 200 the number of probesets starts decreasing at a faster rate (Figure 4.4b), the use of this value appeared optimal for the present experiment. Therefore the cdf file obtained using a threshold intensity of 200 was chosen for undertaking the later transcriptome analysis.

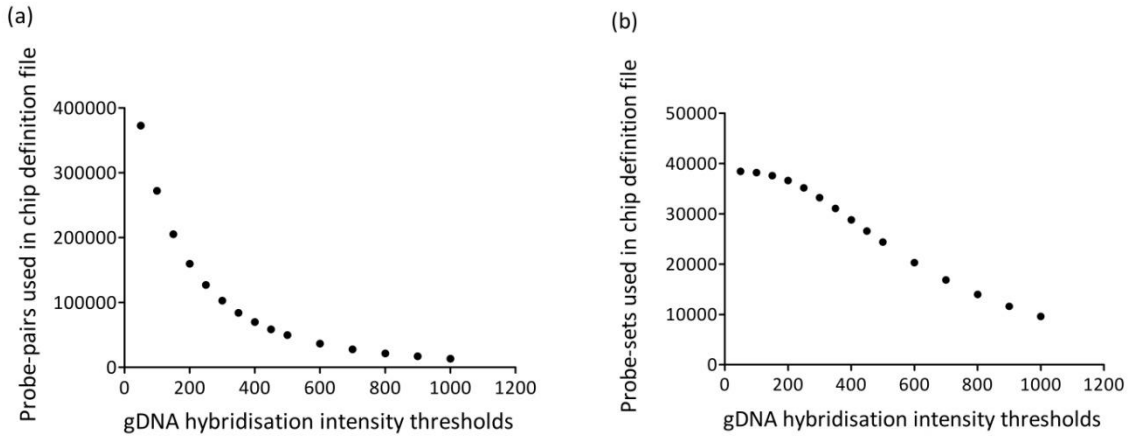

Figure 4.4 Number of probe-pairs (a) and probe-sets (b) used to examine the transcriptome of the Japanese quail, as a function of the quail genomic DNA (gDNA) hybridisation intensity thresholds used to modify the chip definition files.

4.3.15.2 Statistical transcriptome analysis

Prior of analyses, Affymetrix library files were downloaded (http://www.affymetrix.com/support/technical/libraryfilesmain.affx, August 2012). These files provided the chicken genome version galgal3 (Ensembl release 60); the cdf file for GeneChip® Chicken Array was modified as described above (Section 4.3.15.1). Raw data cel files (n = 12) of the RNA hybridised samples were normalised using the GC-content by the Robust Multichip Average method within Genomics Suite (Partek, Saint Louis, USA). Normalised data were quality checked using the available metrics in Partek software. Normalised raw data resulted in a total of 36684 probesets. Probesets lacking an annotation (gene symbol and/or Ensembl Identifier) were filtered out (27.7 % of total probesets) and only the annotated transcripts (26522) were statistically analysed.

Principal Component Analysis (PCA) plots were first performed to assess replicate clustering within each condition. The $\log_2$-transformed normalised signal intensities of annotated transcripts were then analysed using two-way ANOVA to identify the genes that were differentially expressed by the pre- or post-hatching treatment, or their potential interactions. To control for false positives, *p*-values were adjusted for multiple comparisons using false discovery rate (FDR or *q*-value) (Benjamini and Hochberg, 1995); a cut-off of FDR ≤ 0.1 was used to identify differentially expressed genes.

Independently from ANOVA, I also performed the non-parametric RankProducts (Breitling *et al.*, 2004) to analyse differential expression in pair-wise comparisons as described above (section 4.3.12.1), and then compared the data with those obtained from the RNA-seq experiment.

4.3.15.3 Comparison between RNA-seq and Microarrays

In order to achieve an objective comparison between Microarrays and RNA-seq platforms, a common unique set of transcripts between the two platforms were identified using the Ensembl identifiers; all the other non-common transcripts were filtered out. To analyse the correlation between RNA-seq and Microarray

data, I performed non-parametric Spearman-correlation tests between each biological replicate using both common and non-redundant Ensembl identifiers between the two technologies (n = 7172). The redundancies were mainly due to the nature of the Microarray data as there may be multiple probe-sets mapping to the same transcript. In order to compare the potentially differentially expressed genes detected by RankProducts from RNA-seq and Microarray data, I first obtained 6 databases (one per each pair-wise comparison) of common transcripts between the two platforms, and then, I selected the common up- and down-regulated genes using the non-stringent FDR cut-off value of 0.20 (Zheng, 2012).

4.3.16 Hardware specifications

TopHat and Cuffdiff were run on a shared server with 16 cores, 16GB RAM and 24TB hard-disk (Glasgow Polyomics Facility, University of Glasgow, UK). All the other packages used were run on desktop computers with at least 4GB RAM.

4.4 Results

4.4.1 RNA quality control

The RNA quality of all the 48 individuals selected for the RNA-seq experiment was excellent in both the hippocampus and hypothalamus (RINs: mean ± SEM, 9.10 ± 0.06 and 9.34 ± 0.05, respectively). Importantly, storage time did not alter the RNA quality in the RNA hippocampal pools, which was assessed just prior the start of the microarray experiments (RIN: mean ± SEM, 8.90 ± 0.04; see also Figure A2 in the Appendix for the electropherogram images).

4.4.2 Raw data quality control

The Phred quality distributions per sequencing cycle for all the 24 samples can be seen in Figure 4.5. In both the hippocampal and hypothalamic samples the accuracy of the reads was overall high throughout the sequencing runs ($Q \geq 30$). However, as expected from Illumina technology, the error score associated to the base-calls in each cycle tended to increase after approximately 30-35 cycles.

4.4.3 Read Alignment

I extracted key alignment metrics from the TopHat outputs, as shown in Table 4.2. As can be seen, between 56% and 62% of reads mapped to the reference genome. After correcting for multiple mapping, I found between 52% and 57% unique hits to the reference genome. The number of reads spanning the predicted splice junctions varied between 856832 and 1552242, which corresponded to 6.4-7.2% of all the mapped reads.

Table 4.2 Alignment basic statistics across the 3 biological replicates in each treatment (CC, BC, CB and BB) in (a) hippocampus and (b) hypothalamus. Data were expressed as % relatively to the total number of sequenced reads. The latter information was extracted from the FastQC summary outputs.

a) Hippocampus

Treatment	CC			BC			CB			BB		
Replicate	1	2	3	1	2	3	1	2	3	1	2	3
Total sequenced reads	27148903	25184438	27291964	29301542	28935750	30601746	28535217	28846613	32629108	29029598	28663520	30836664
Total reads mapped (%)	59.81	58.36	59.33	59.36	59.39	58.58	60.35	60.06	61.01	58.89	59.18	58.55
Reads uniquely mapped to the reference genome (%)	56.19	54.78	55.67	55.74	55.77	54.96	56.69	56.33	57.38	55.24	55.51	54.81
Total splice junctions	84287	81745	82718	84565	83066	84976	83868	81840	86524	84951	80711	84946
Total reads spanning the junctions	1122435	988787	1090408	1208801	1138149	1234046	1201800	1145576	1379557	1157604	1117586	1171845

b) Hypothalamus

Treatment	CC			BC			CB			BB		
Replicate	1	2	3	1	2	3	1	2	3	1	2	3
Total sequenced reads	33923472	34308219	28910969	26728956	32415075	25984762	36565661	30406958	24037791	25667973	28399658	26341867
Total reads mapped (%)	61.60	61.54	58.12	59.25	62.02	59.68	60.95	60.19	55.94	59.71	60.70	59.92
Reads uniquely mapped to the reference genome (%)	57.78	57.65	54.28	55.60	58.16	55.96	57.28	56.27	52.31	55.85	56.95	56.11
Total splice junctions	91215	91538	83414	84961	90430	83371	92346	86538	76735	82233	86088	84032
Total reads spanning the junctions	1444205	1501664	1116771	1131181	1447955	1069989	1552242	1244302	856832	1067798	1213705	1103543

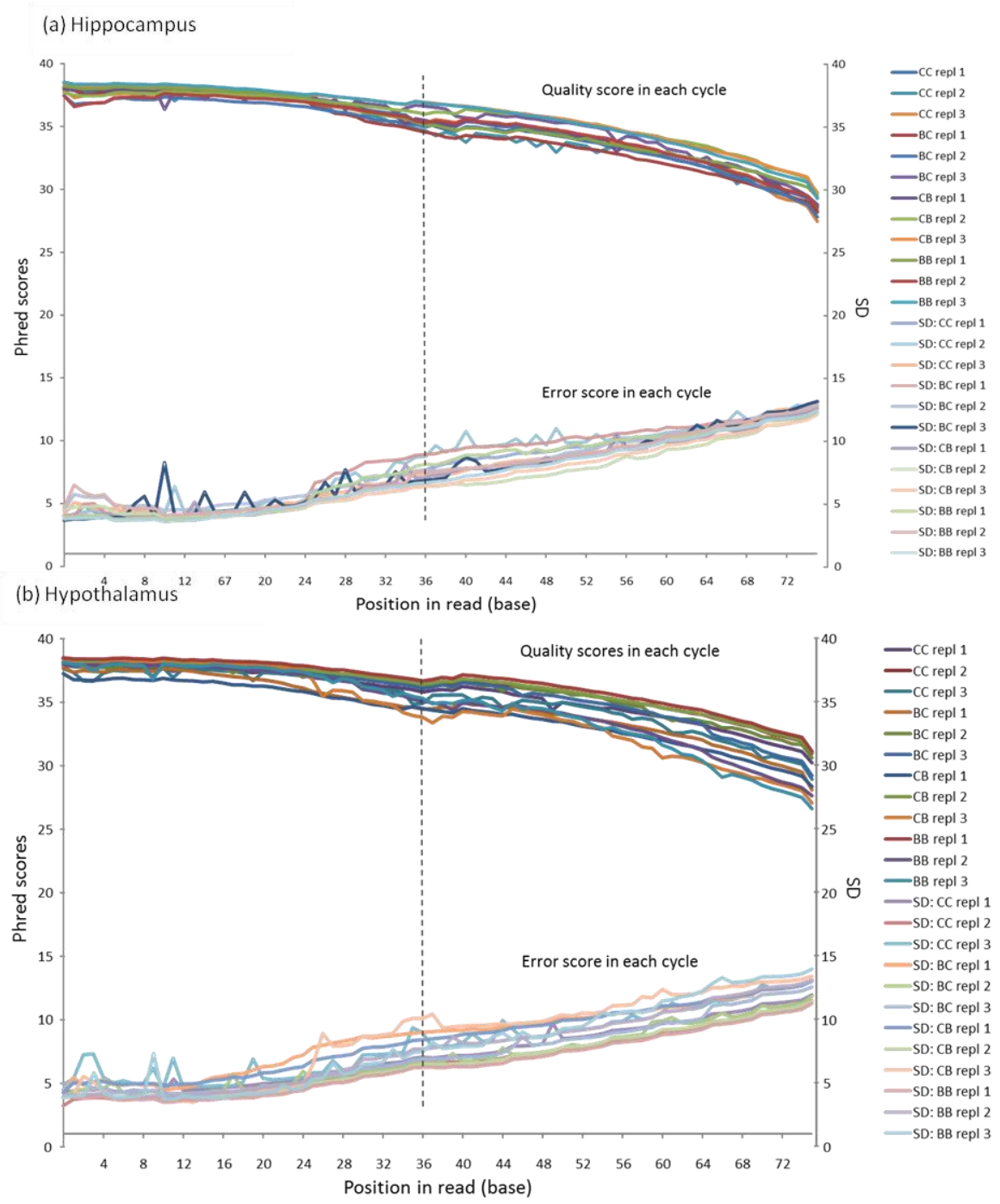

Figure 4.5 Plots showing the average Illumina quality Phred scores (estimating the accuracy of the reads) and their corresponding standard deviations (referred as "SD in the legend and estimating the error score) in each cycle per base-call along the 76 bases reads in the (a) hippocampus (n = 12) and (b) hypothalamus (n = 12) across the three pooled biological replicates (repl 1, repl 2 and repl 3) in each experimental treatment group (CC, BC, CB, or BB). The values were calculated using a Perl script written by Pawel Herzyk. The dotted line at the level of the 36th base represents the length chosen to trim the reads that were then used in the further analyses as after this sequencing cycle the quality of the cycle per base-call tended to decrease consistently across all the samples.

4.4.4 PCA

The PCA plot of all the RNA-seq samples clearly shows 2 tissue-specific expression patterns along the PC1, which explained 28.1% of the global variation (Figure 4.6).

The PCA plot with only the hippocampal samples showed a larger variation in the BB and CB samples in comparison with the CC and BC samples (Fig 4.7a). In the hypothalamus, replicate samples within the CC, BC or BB treatment showed a good clustering among each other, while high within-treatment variability was detected in the CB samples (Figure 4.7b). Such high within-treatment variation is likely to represent true biological variation (due to the RNA pooling approach), rather than technical biases of the RNA-seq analysis (see Paragraph 4.5.2 for a discussion on this aspect). This complicating factor was taken into consideration and minimised in the differential gene expression statistical analysis by filtering out the data that showed inconsistent gene expression responses within the treatment biological replicates according to the specific biological questions of this study (see Paragraphs 4.4.5.2, 4.4.6, and 4.4.7).

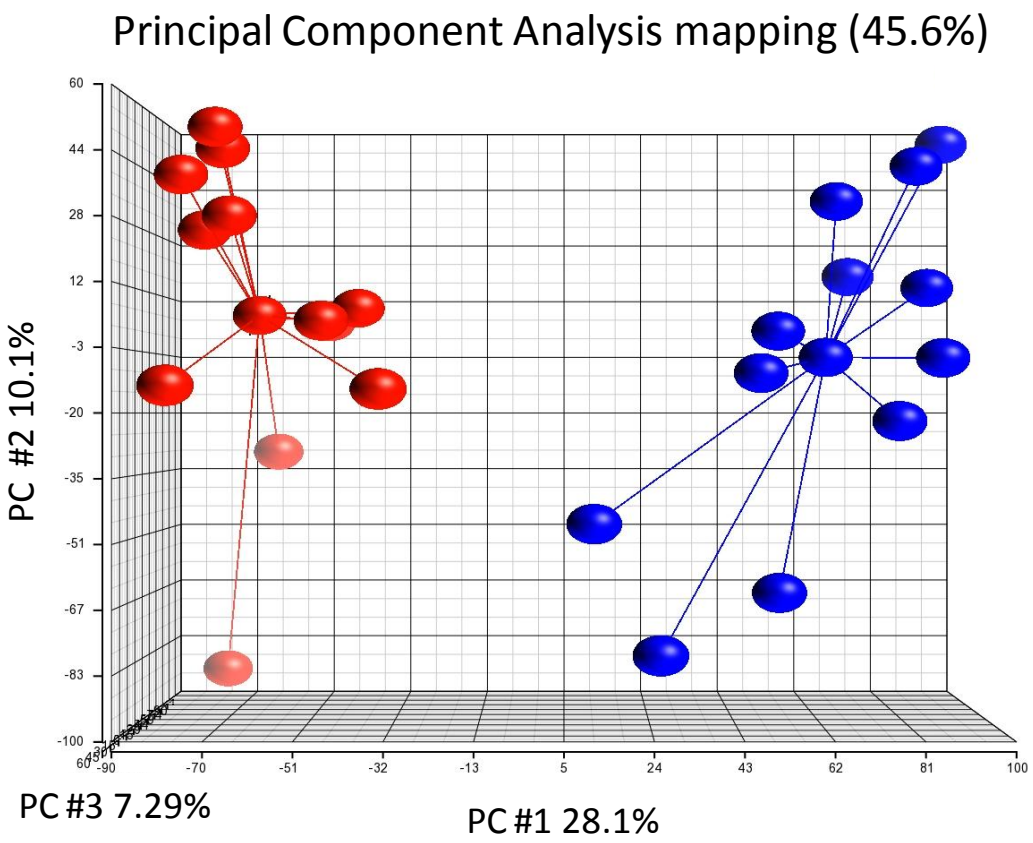

Figure 4.6 Principal Component Analysis plot (PCA) of all RNA-seq sample (n = 24) using the FPKM produced by Cufflinks (n = 17914 transcripts). Data are clustered by tissue type using the centroid function, regardless of the treatment groups. Red circles: hippocampal samples; blue circles: hypothalamic samples. PC #1 = first component, explaining 28.1% of the variation across genes; PC #2 = second component, explaining 10.1% of the variation across genes, and PC #3 = third component, explaining 7.29% of the variation across genes. Similar PCA plot was obtained using the RNA-seq count data obtained by HT-Seq (data not shown).

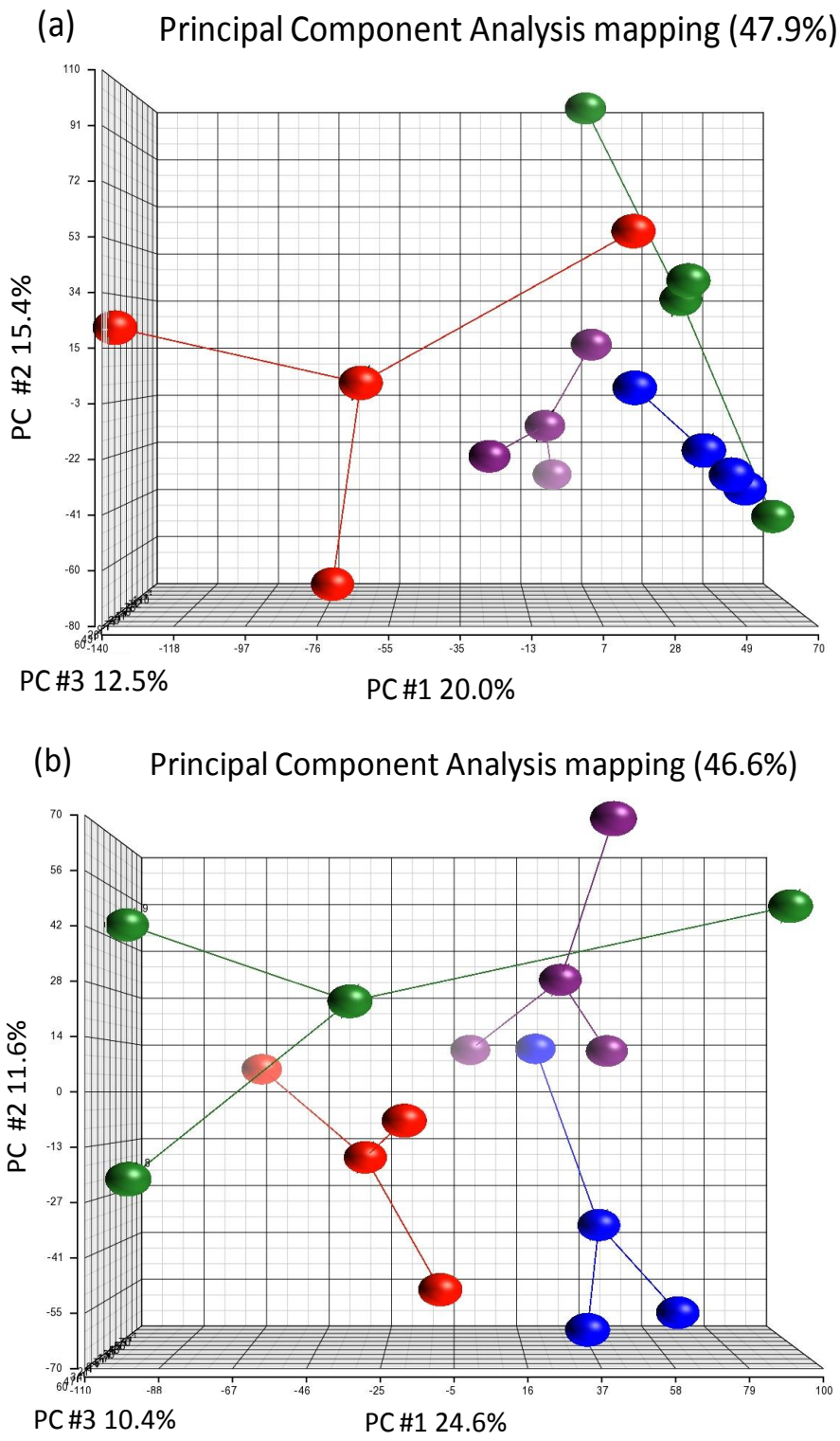

Figure 4.7 Principal Component Analysis (PCA) plots of RNA-seq samples in the (a) hippocampal samples (n = 12) and (b) hypothalamic samples (n = 12) using the FPKM produced by Cufflinks (n = 17914 transcripts). Data are clustered by treatment groups (using the centroid function). Purple circles = CC; blue circles = BC; green circles = CB, and red circles = BB. PC #1 = first component, PC #2 = second component; PC #3 = third component; % values measure the variation explained by each component. Similar PCA plots were obtained using the count data obtained using HT-Seq (data not shown).

4.4.5 Pilot study: RNA-seq intra-platform statistical comparison

4.4.5.1 Bayseq multi-factorial models

In the hippocampus 4 out of 14 DE models showed genes with FDR ≤ 0.20 for a total number of 32 genes, whereas in the hypothalamus only 1 out of 14 DE models showed 2 genes with FDR ≤ 0.20 (Table 4.3). Annotation of these genes and statistics is reported in Table A2 and A3 (Appendix); as can be noticed, there was a high biological variation. The top- differentially expressed genes (FRD: 0.05-0.1) tended to have low count scores (i.e. counts < 5) and are likely to be false positives (Table A2).

Table 4.3 Number of genes with FRD ≤ 0.20 in Bayseq models (DE). Treatment groups in brackets define group of samples with the same distribution. In each treatment group there are 3 biological replicates.

Model name	Model description	Tissue	Genes FRD ≤ 0.20
DE2	(CC, BC, CB) (BB)	hippocampus	11
DE4	(BC, BB, CB) (CC)	hippocampus	9
DE6	(CC, BB) (BC, CB)	hippocampus	3
DE7	(CC, CB) (BC, BB)	hippocampus	9
DE5	(CC, BC) (BB, CB)	hypothalamus	2

4.4.5.2 Differential expression pair-wise analyses using Cufflinks, Bayseq and RankProducts

The total number of genes at FDR ≤ 0.20 across Cufflinks, Bayseq and RankProducts are shown in Table 4.4. As can be seen, RankProducts showed a tendency to capture larger number of genes, followed closely by Cufflinks whilst Bayseq found far less genes among the majority of the contrasts. Furthermore, the concordance between Cufflinks and RankProducts was high with 80.26 ± 14.73% (mean ± SD) of shared genes in the hippocampus and 79.78 ± 18.95% (mean ± SD) of common genes in the hypothalamus (Figures 4.8-4.11). Full details of the genes that were differentially expressed across all the three methods in each contrast are presented in Table A4 (Appendix).

In the hippocampus, the contrast BC *vs* CC is of particular interest because it showed the most enriched gene lists in Cufflinks, Bayseq and RankProducts with, respectively, 18 and 41 up- and down-regulated genes across all three methods (corresponding respectively to 58.57% and 39.13% of the genes detected by Bayseq) (Figure 4.8).

Table 4.4 Total number of differentially expressed genes at FDR ≤ 0.20 across Cufflinks, Bayseq and RankProducts in the (a) hippocampus and (b) hypothalamus. Arrows indicate gene expression directional changes (down- or up-regulation) and refers to the 2nd class vs 1st class.

(a) Hippocampus

Contrast	Cufflinks		Bayseq		RankProducts	
(2nd class vs 1st class)	↓	↑	↓	↑	↓	↑
BC vs CC	180	80	70	46	349	324
CB vs CC	45	9	1	0	79	5
BB vs CC	90	52	13	2	185	75
CB vs BC	6	37	0	1	29	13
BB vs BC	32	141	5	0	172	142
BB vs CB	33	99	0	0	7	97

Contrast	Cufflinks		Bayseq		RankProducts	
(2nd class vs 1st class)	↓	↑	↓	↑	↓	↑
BC vs CC	32	37	0	1	32	56
CB vs CC	6	43	0	0	15	42
BB vs CC	20	86	3	63	68	263
CB vs BC	14	32	0	0	53	54
BB vs BC	15	64	2	19	37	188
BB vs CB	6	1	0	0	3	13

The RankProducts down-regulated gene list from the BC *vs* CC comparison included most of the genes that were detected by Cufflinks and Bayseq (95.5% and 92.8%, respectively) plus 43.8% more genes; similarly, RankProducts up-regulated gene list in the same contrast included 93.4% and 92.5% of the genes

captured respectively by Cufflinks and Bayseq, and added 68.51% more genes. Importantly, among the relevant up-regulated genes there were those coding the MR receptor (nuclear receptor subfamily 3, group C, member 2) and the G-protein-coupled estrogen receptor; while among the top down-regulated genes there were somatostatin II; proenkephalin; transthyretin; superoxide dismutase 3, extracellular; growth hormone regulated TBC protein 1 (Table A4a, Appendix).

In the hypothalamus, the contrast BB *vs* CC showed the mostly populated gene lists across the three methods and the expression differences were skewed towards an up-regulation: 43 up-regulated genes in the BB birds compared to the CC birds were shared among Cufflinks, Bayseq and RankProducts (corresponding to 68.2% of the total genes captured by Bayseq; Figure 4.10). Again, RankProducts appeared to be the least conservative by including 100% and 96.8% of the genes found by Cufflinks and Bayseq, respectively, and found 159 more genes (Figure 4.10). Of particular interest is the significantly higher expression detected by the three packages of two types of serotonin receptors (5-hydroxytryptomine receptor 2C and 5-hydroxytryptomine receptor 3A) in the pre- and post-hatching B-exposed birds compared with the controls (Table A4b, Appendix).

In summary, the comparison across the three statistical methods within each pair-wise contrasts suggested that RankProducts was the best statistic due to the limited number of biological replicates for each treatment; large intra-replicate variation within each treatment and the overall good reproducibility with Cufflinks data, showing on average approximately 80% of overlapping genes in both the hippocampal and hypothalamic tissues between these two packages. RankProducts showed higher sensitivity than Cufflinks and Bayseq, consistently detecting higher numbers of differentially expressed candidate genes in almost all comparisons. However, in the awareness that the larger number of genes detected by RankProducts might also include a higher proportion of false positives, RankProducts data were further filtered using Vector Analysis (Breitling *et al.*, 2005) as explained in detail below (Section, 4.4.7). To some extent the use of Vector Analysis also allowed me to overcome the limitation due to the pair-wise comparison approach.

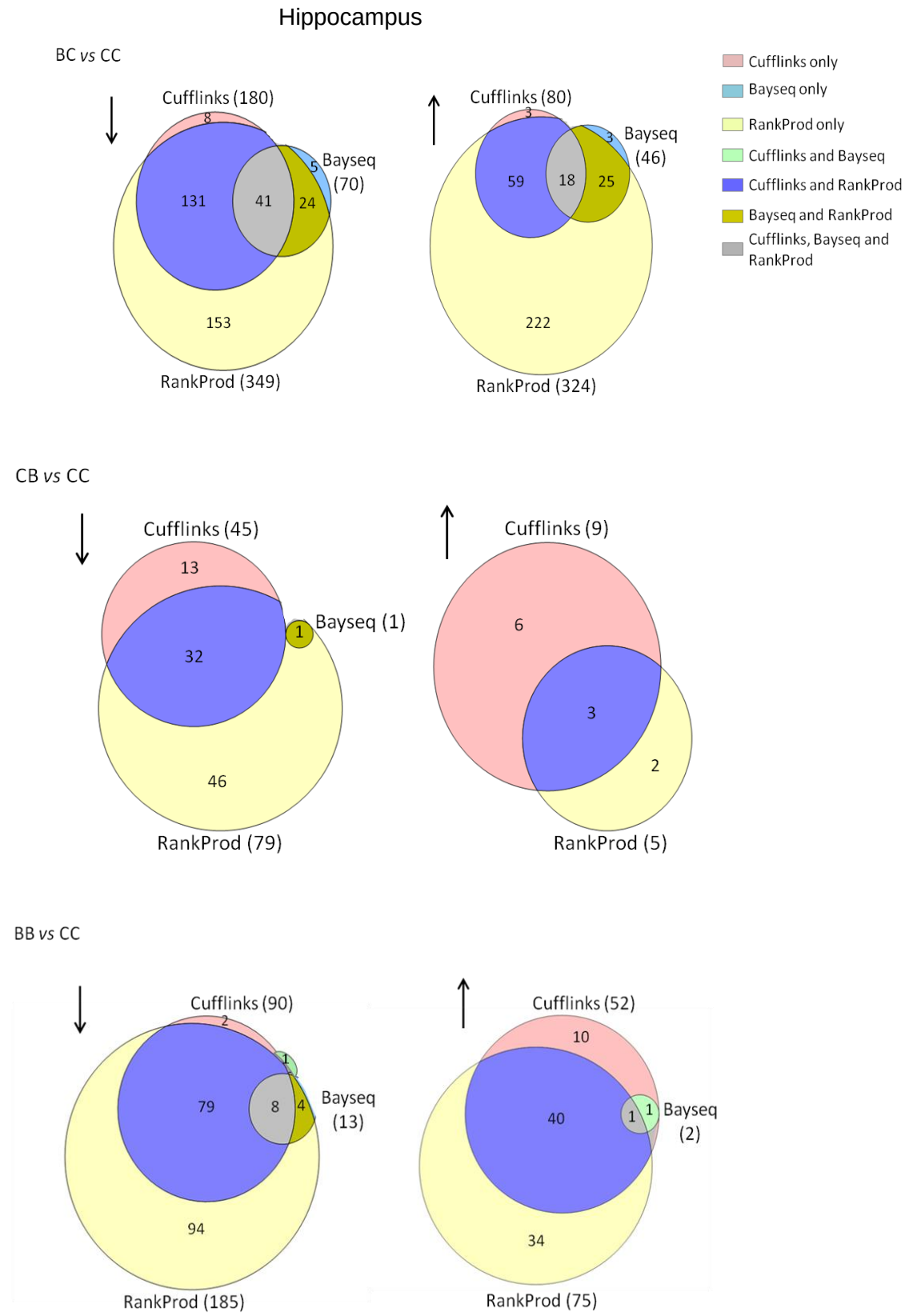

Figure 4.8 Proportional Venn Diagrams showing all the number of genes within the hippocampus in common across Cufflinks, Bayseq, and RankProducts (indicated as RankProd) at FDR ≤ 0.20 in the contrasts BC *vs* CC, CB *vs* CC, and BB *vs* CC. Comparisons were performed separately for down- and up-regulated genes (as indicated by the arrows); gene expression directional changes refer to the 2nd class vs 1st class.

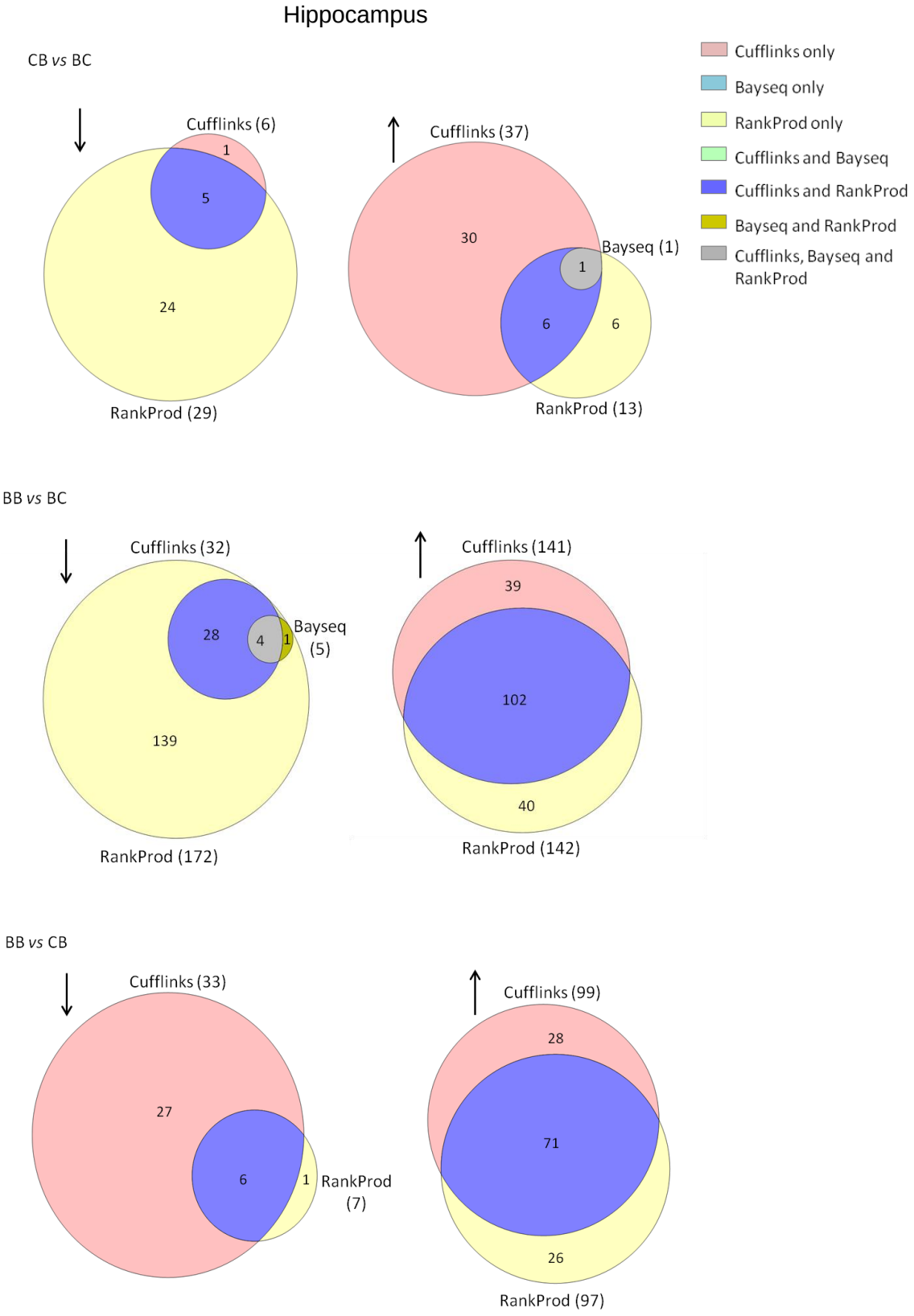

Figure 4.9 Proportional Venn Diagrams showing all the number of genes within the hippocampus in common across Cufflinks, Bayseq, and RankProducts (indicated as RankProd) at FDR ≤ 0.20 in the contrasts CB *vs* BC, BB *vs* BC, and BB *vs* CB. Comparisons were performed separately for down- and up-regulated genes (as indicated by the arrows); gene expression directional changes refer to the 2nd class vs 1st class

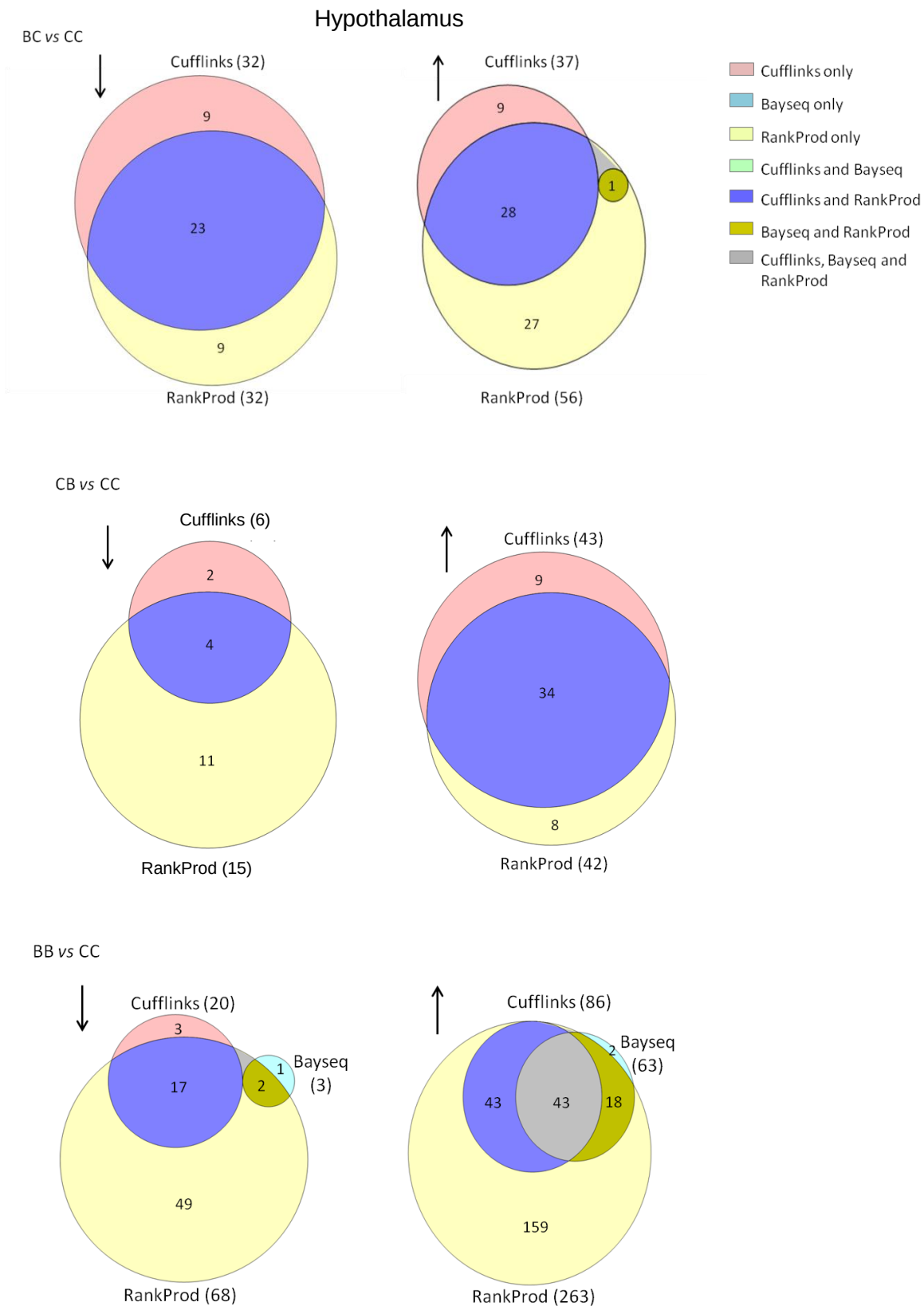

Figure 4.10 Proportional Venn Diagrams showing all the number of genes within the hypothalamus in common across Cufflinks, Bayseq, and RankProducts (indicated as RankProd) at FDR ≤ 0.20 in the contrasts BC *vs* CC, CB *vs* CC, and BB *vs* CC. Comparisons were performed separately for down- and up-regulated genes (as indicated by the arrows); gene expression directional changes refer to the 2nd class vs 1st class

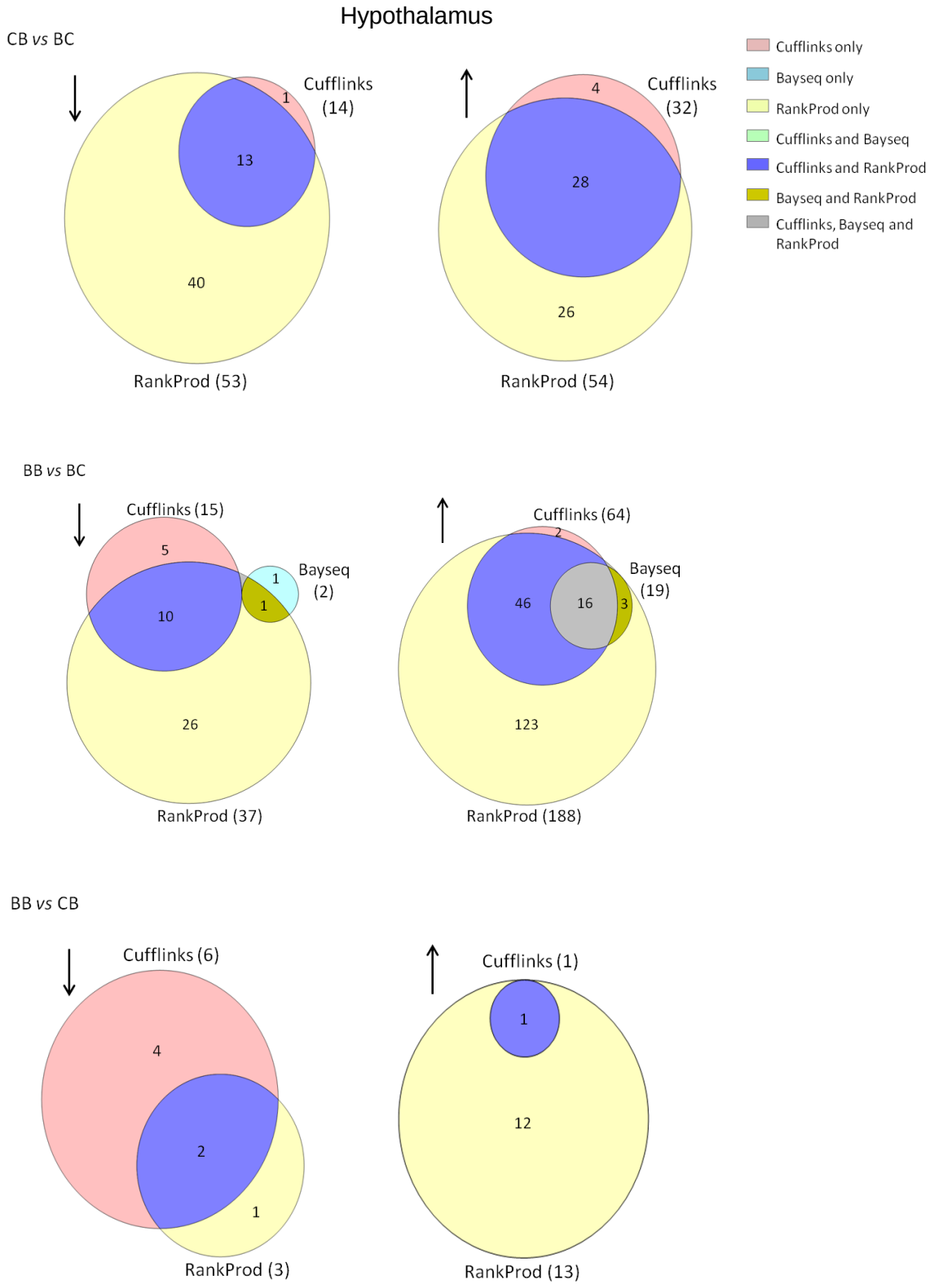

Figure 4.11 Proportional Venn Diagrams showing all the number of genes within the hypothalamus in common across Cufflinks, Bayseq, and RankProducts (indicated as RankProd) at FDR ≤ 0.20 in the contrasts CB *vs* BC, BB *vs* BC, and BB *vs* CB. Comparisons were performed separately for down- and up-regulated genes (as indicated by the arrows); gene expression directional changes refer to the 2nd class vs 1st class.

4.4.6 RankProducts analysis

The number of significant candidate genes revealed by RankProducts across the pair-wise contrasts in both the hippocampal and hypothalamic samples is shown in Table 4.5.

Table 4.5 Number of significant transcripts (FDR ≤ 0.10) that were up- or down- regulated across the pair-wise comparisons in the (a) hippocampus and (b) hypothalamus.

(a) Hippocampus

Contrast: (2^{nd} class vs 1^{st} class)	up-regulated genes under 2^{nd} class	down-regulated genes under 2^{nd} class
BC vs CC	159	231
CB vs CC	3	21
BB vs CC	19	127
CB vs BC	4	10
BB vs BC	53	43
BB vs CB	43	2

(b) Hypothalamus

Contrast: (2^{nd} class vs 1^{st} class)	up-regulated genes under 2^{nd} class	down-regulated genes under 2^{nd} class
BC vs CC	56	32
CB vs CC	21	5
BB vs CC	159	22
CB vs BC	24	31
BB vs BC	117	19
BB vs CB	10	3

As can be seen, overall, RankProducts detected a higher number of candidate genes in the hippocampus than in the hypothalamus. The top 20 up-and down-regulated genes per each comparison in both the tissues are shown in Table A5 (Appendix). Specifically, in the hippocampus, the birds exposed to B only pre-hatching showed the highest number of differentially expressed genes when compared with the adult controls; the differences were skewed towards a repression of gene expression with the fold changes for the down-regulated genes ranging from -18.2 to -1.5, whilst the fold changes for the up-regulated

significant genes ranging from 1.9 to 1.4 (Table A5a, Appendix). The birds that were treated both pre- and post-hatching also showed several down-regulated genes (fold change ranged from -28.1 to -1.6; Table A5a, Appendix) when compared to the control individuals and among the top down-regulated genes there were transthyretin, arrestin, and extracellular-superoxide dismutase 3 similar to what was observed in the BC *vs* CC comparison. In the hypothalamic samples, the comparisons between BB *vs* CC and BB *vs* BC showed the highest number of significantly expressed candidate genes and, in contrast with what was observed in the hippocampus, the transcriptome differences were skewed towards an overall increase in gene expression with fold changes ranging from 3.3 to 1.4 (Table A5b, Appendix).

4.4.7 Vector analysis and behavioural categories

A graphical summary and the number of genes that were filtered according to the behavioural categories using the Vector Analysis (Section 4.3.13.1) are reported in Figures 4.12-4.16 and Table 4.6 (in Table A6 in the Appendix is shown the complete lists of genes for each category).

Table 4.6 Number of genes belonging to each behavioural category (see Section 4.3.13.1 for full methodological details) in the hippocampal and hypothalamic samples.

Behavioural category		Hippocampus	Hypothalamus
I.	Pre- and post-hatching B responsive genes	62	19
II.	Pre-hatching B responsive genes	29	14
III.	Post-hatching B responsive genes	0	88
IV.	Pre- and post-hatching B responsive genes: "*cumulative effect*"	30	3
V.	Interacting pre- and post-hatching B responsive genes: "*null effect*" via elevating expression in BC and reducing	0	5
VI.	Interacting pre- and post-hatching B responsive genes: "*null effect*" via elevating expression in CB and reducing	1	2

4.4.7.1 Hippocampus

Consistently with the pair-wise RankProducts analysis, the overall long-lasting effects induced by the pre-hatching exposure to B in the hippocampus were stronger than those induced by post-hatching B (Figures 4.12-4.13). In fact, while no genes were specifically regulated by post-hatching B alone, 29 genes (5.92% of the total 490 genes) were detected as specifically regulated by pre-hatching B (Figure 4.12-II). The changes observed in these pre-hatching B sensitive genes were strongly skewed towards a repression of their expression, with 28 genes down-regulated and only 1 gene up-regulated. Moreover, there were 62 genes (12.65% of the total) that were affected by the overall effect of pre- and post-hatching B, regardless of the timing of exposure, meaning that in such genes the abundance values across the B-treated birds (i.e. BC, CB and BB) were similar, but significantly different when compared to the control birds (Figure 4.12-I). Similarly as before, the changes in transcript abundance of such genes were skewed towards a down-regulation (49 genes were down-regulated and 13 were up-regulated). Importantly, the gene encoding the MR receptor (NR3C2) and not the GR receptor (NR3C1) was significantly up-regulated in the B-phenotypes compared to the controls (Figure 4.14; Table A6, Appendix). In 30 transcripts (6.12% of the total) the long-term effects of pre- and post-hatching B were "*cumulative*" in the birds that experienced both the protocols: 28 genes were down-regulated and only 2 genes up-regulated (Figure 4.13-IV). Interestingly, the pre- and post-hatching treatment induced opposite effects on AVT, with post-hatching B elevating its expression and pre-hatching B decreasing it (Figure 4.13-VI). As result of this opposite interaction the expression values in the BB birds were similar to those observed in the control ("*null effect*").

Effects of post-hatching B given the pre-hatching environments

Effects of pre-hatching B given the post-hatching environments

Hippocampus

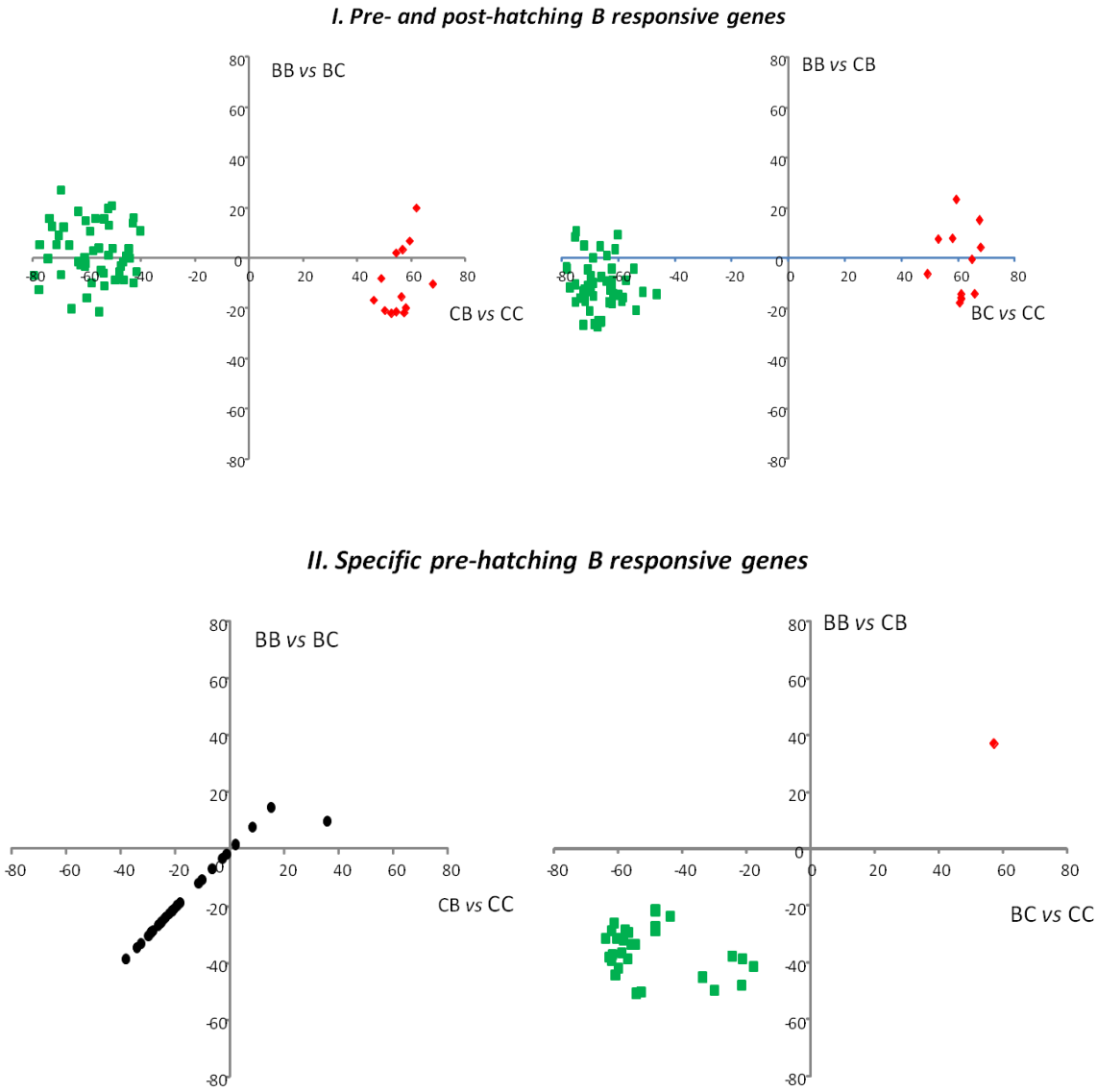

Figure 4.12 Up-regulated (red) and down-regulated (green) genes within the hippocampus filtered according to the behavioural categories I and II described in full detail in the Section 4.3.13.1.

Effects of post-hatching B given the pre-hatching environments

Effects of pre-hatching B given the post-hatching environments

Hippocampus

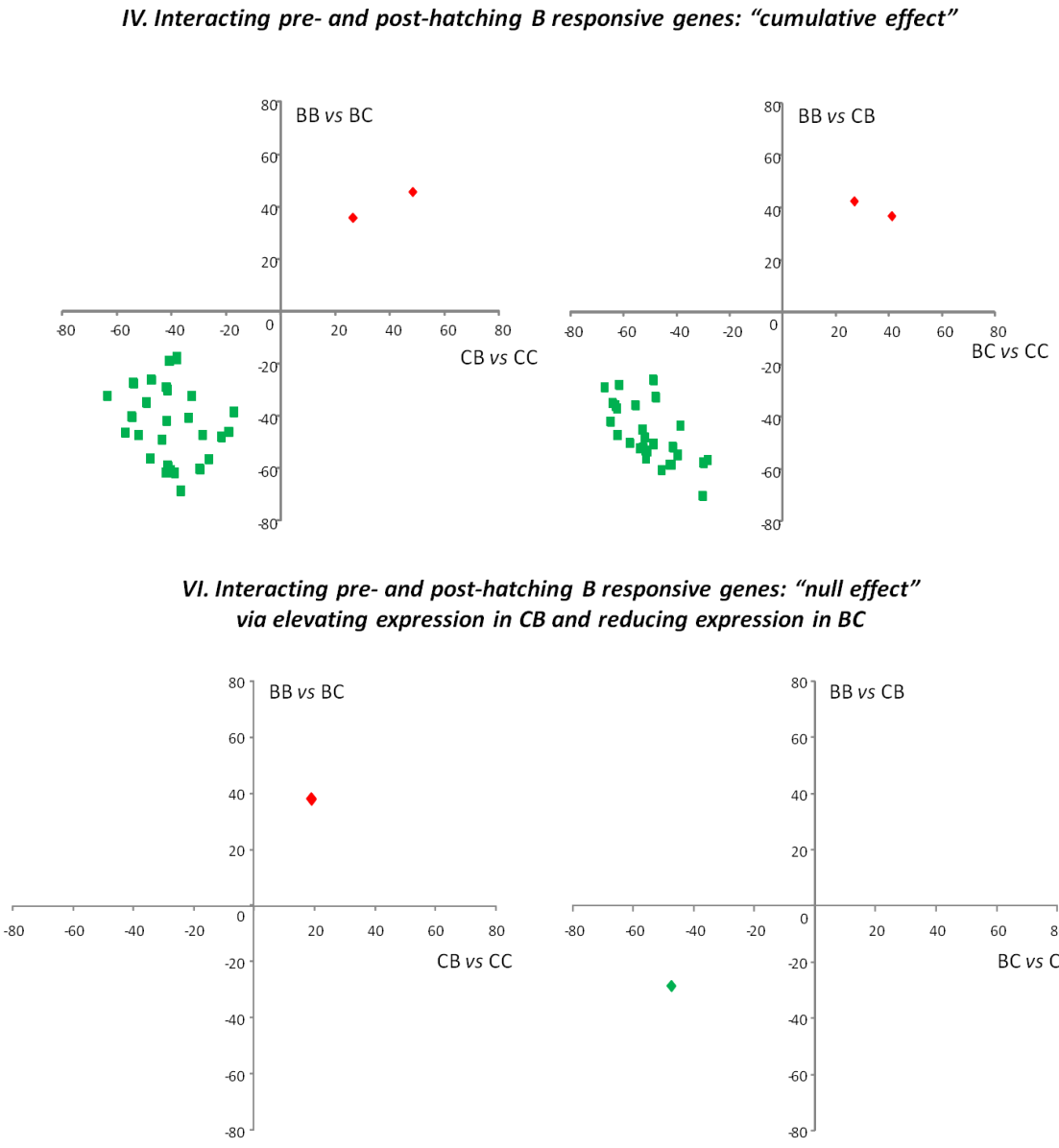

Figure 4.13 Up-regulated (red) and down-regulated (green) genes within the hippocampus filtered according to the behavioural categories IV and VI described in full detail in the Section 4.3.13.1.

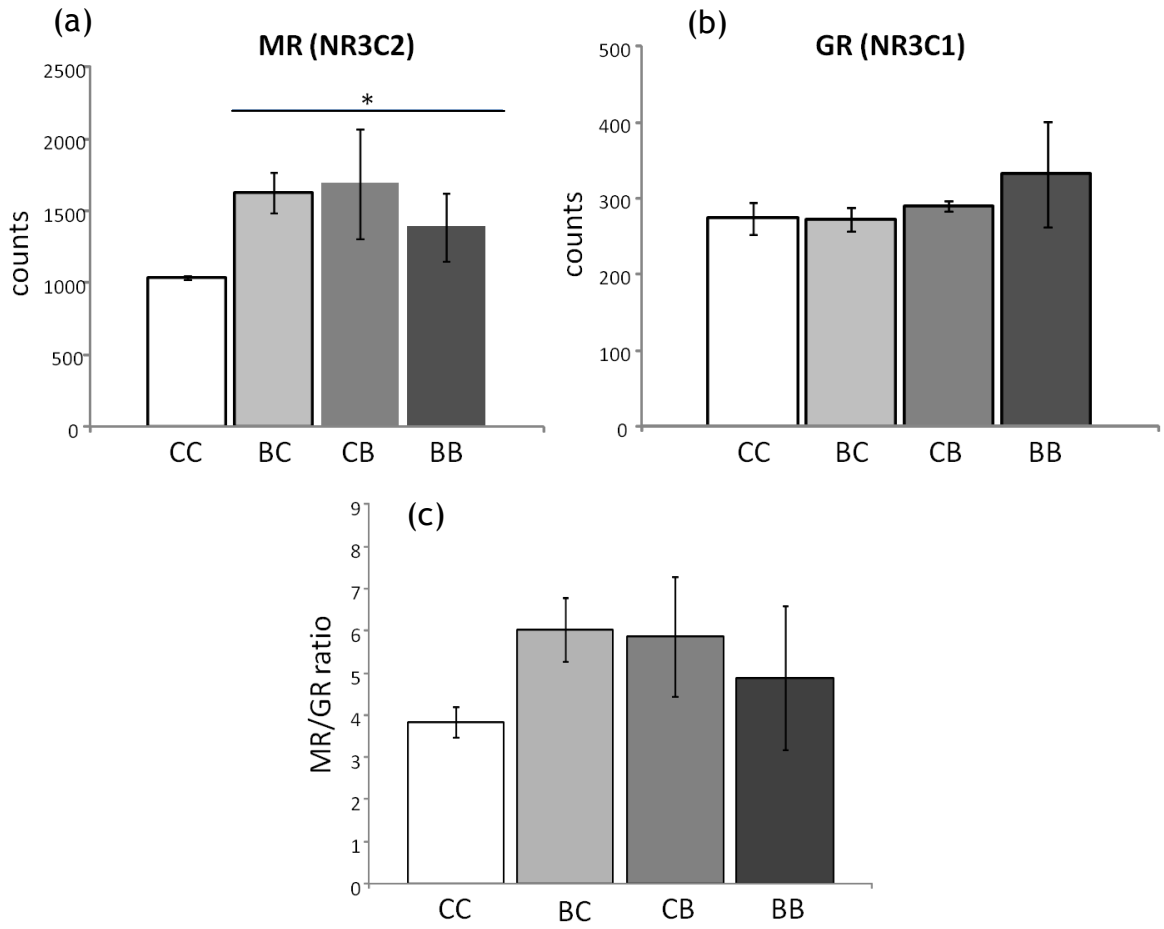

Figure 4.14 Expression values (counts) of the gene coding (a) the mineralocorticoid receptor or MR (NR3C2), (b) the glucocorticoid receptor or GR (NR3C1), and (c) the comparison of the MR/GR expression ratio across the treatment groups (CC, BC, CB, and BB) in the hippocampal samples. In (a) * indicates significant differential expression (behavioural category I, see also Figures 4.2-I and 4.12-I).

4.4.7.2 Hypothalamus

In contrast with what was observed in the hippocampus, in the hypothalamus the magnitude of the long-term effects of post-hatching B was higher than that caused by pre-hatching B and, overall, the genes' dynamic changes across the post-hatching B-treated birds were skewed towards an up-regulation (Figures 4.15-4.16). In fact, the expression of 88 hypothalamic genes (22.64% of the total 302 transcripts; 85 and 3 genes were up- and down-regulated, respectively) was specifically modulated by B administered during the post-hatching development (Figure 4.15-III), whereas only 14 transcripts (4.63% of the total, 10 and 4 genes were up- and down-regulated, respectively) were specifically regulated by the exposure to B *in ovo* (Figure 4.15-II). Only a few number of genes (n = 19, of

which 12 were up-regulated and 7 down-regulated; 6.29% of the total) showed the same dynamic responses to the pre- and post-hatching B protocols when compared to the controls (Figure 4.15-I). As shown in Figure 4.16-IV, cumulative interacting changes in the birds that experienced the combined stress exposure were identified in 3 transcripts (0.99%), of which 2 (C-type lectin domain family 3; member B and similar to protocadherin gamma C5) and 1 (ENSGALG00000023036, not annotated) were respectively up- and down-regulated. The negative interactions of pre- and post-hatching B were seen in 7 genes (2.32% of the total) with 5 genes (pappalysin 2; similar to neuropilin-2a1 receptor; neuropilin 2; adhesion molecule with Ig-like domain 2; ENSGALG00000016258 (not annotated); chloride intracellular channel 5) showing a "*null effect*" via up-regulation of pre-hatching B and down-regulation of post-hatching B and (Figure 4.16-V), and 2 genes (complement component 1, q subcomponent-like 4; ENSGALG00000024011 (not annotated)) showing a "*null effect*" via up-regulation of post-hatching B and down-regulation of pre-hatching B (Figure 4.16-VI).

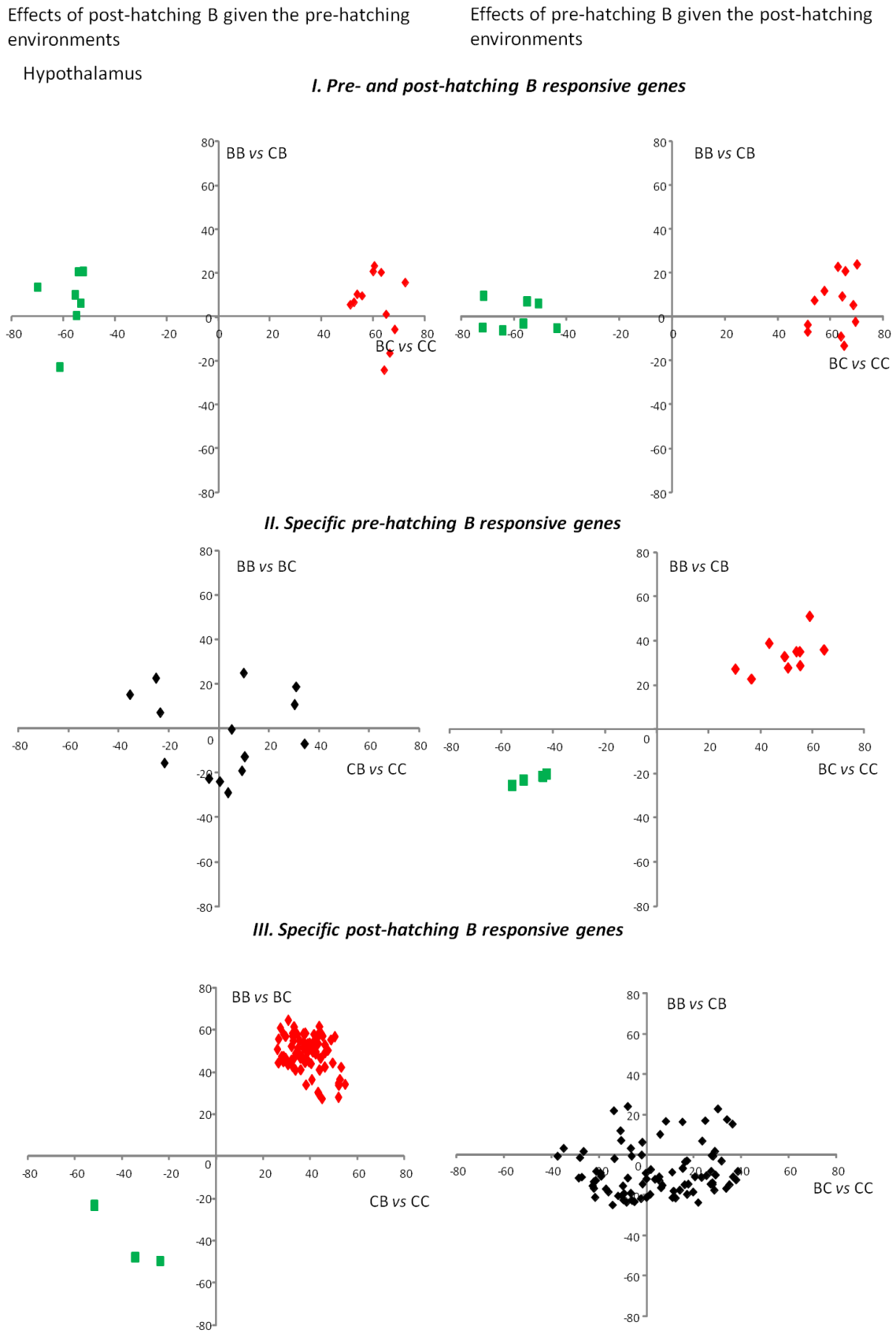

Figure 4.15 Up-regulated (red) and down-regulated (green) genes within the hypothalamus filtered according to the behavioural categories I, II and III described in full detail in the Section 4.3.13.1.

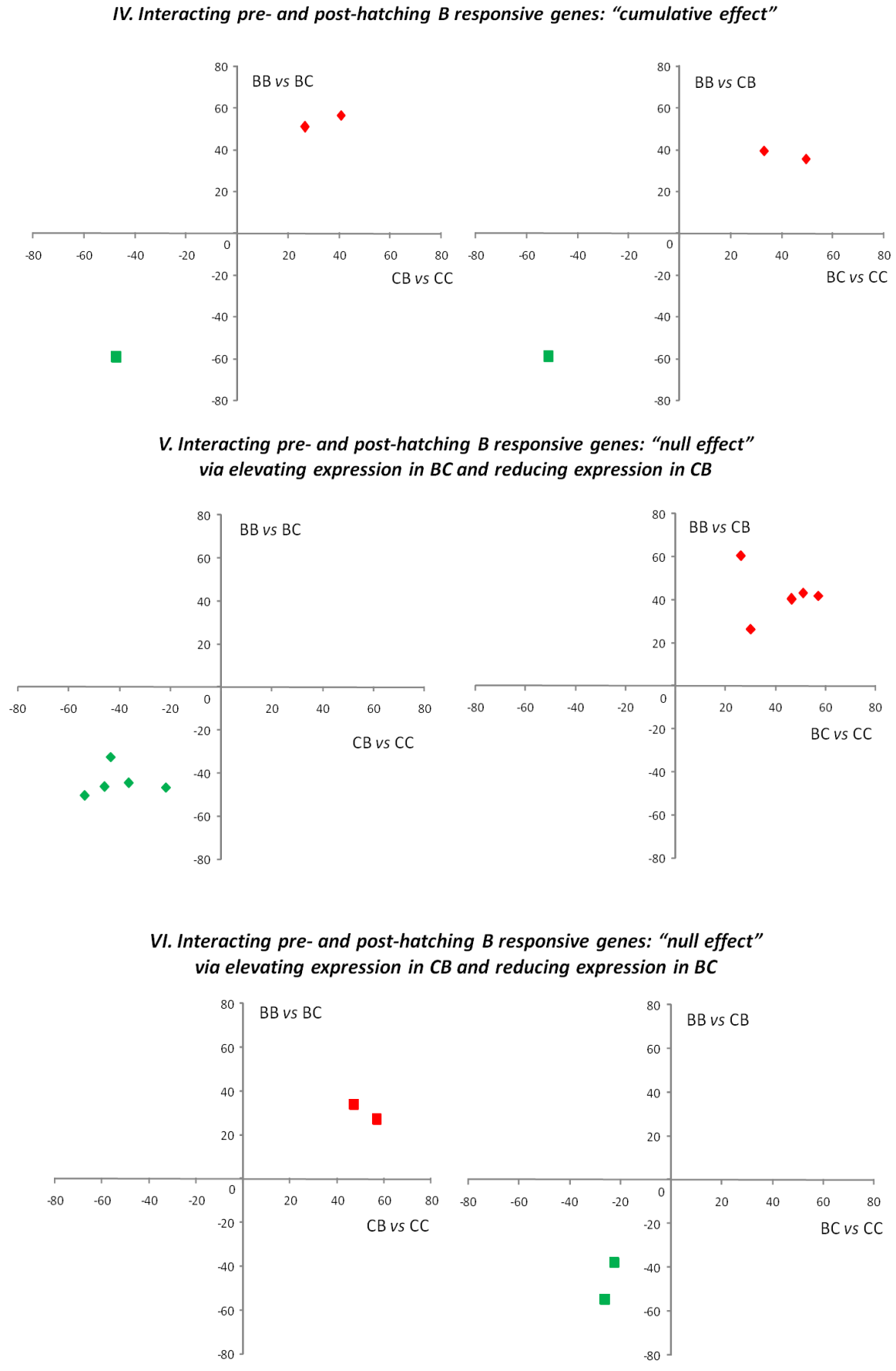

Figure 4.16 Up-regulated (red) and down-regulated (green) genes within the hypothalamus filtered according to the behavioural categories IV, V and VI described in full detail in the Section 4.3.13.1.

4.4.8 Functional analysis

4.4.8.1 Hippocampus

Pre- and post-hatching B responsive genes (category I, Figures 4.2-I and 4.12-I**).** The submission to the IPA server of 62 pre- and post-hatching B responsive genes resulted in successful mapping of 48 genes (38 down- and 10 up-regulated genes, respectively), and of these, 45 were considered as non-redundant "focus" genes with records in the IPA database (Table A7a, Appendix). The significant biological functions associated with the long-term effects of pre- and post-hatching B are shown in Table A8a (Appendix). The most significant biological category was associated with "Renal and Urological Disease" and included the gene coding the MR receptor (NR3C2, see also Figure 4.14) as well as arginine vasopressin receptor AVPR2 (mammalian homolog of AVTR2 in birds – IPA analysis is based on mammalian genomic findings as explained in Paragraph 4.3.14). The most enriched significant biological function was "Nervous System Development and Function" with 14 focus genes, including the brain-derived neurotrophic factor (BDNF), NR3C2, superoxide dismutase 3 (SOD3), neuronal differentiation 6 (NEUROD6), Ca++ - dependent secretion activator 2 (CADPS2), and agrin (AGRN). The genes BDNF, NR3C2 and CADPS2 are known modulator of several stress-related behaviours, such as spatial memory and exploratory behaviour (Table A8a, Appendix). The network analysis revealed the existence of 6 networks with the scores between 30 and 3, which together contained 43 out of 45 genes. The top network (Figure 4.17) contained 14 "focus" genes and included NR3C2, BDNF, SOD3, AVPR2, tyrosinase-related protein 1 (TYRP1), and TIMP metallopeptidase inhibitor 3 (TIMP3). The most significant canonical pathway identified was Superoxide Radicals Degradation with SOD3 and TYRP1 (p = 9.15E-05, ratio = 0.25), which are both involved in oxidation-reduction processes. Interestingly, the upstream analysis identified the hormone B among the top 5 regulators (p = 7.02E-04) for the target genes BDNF, NR3C2 and secreted frizzled-related protein 1 (SFRP1).

Pre-hatching B responsive genes (category II, Figures 4.2-II and 4.12-II)**.** Out of 29 pre-hatching B responsive genes, 25 were successfully mapped to IPA (23 genes were "focus" genes) and are shown in Table A7a (Appendix). All the significant biological functions linked with the specific long-term effects induced by exposure to B *in ovo* are shown in Table A8a (Appendix). The top significant biological function was associated with "Endocrine System Development and Function" and included cytochrome P450, family 19, subfamily A, polypeptide 1 or aromatase (CYP19A1); melanocortin 4 receptor (MC4R); transthyretin (TTR), and Wolfram syndrome 1 (wolframin) or WFS1. The most enriched biological function belonged to the class "Molecular Transport" and included TTR, CYP19A1, MC4R, proenkephalin (PENK), and melanocortin 5 receptor (MC5R). The network analysis found 3 networks with the scores ranging from 27 to 2, which contained 22 genes out of 25. The top network (Figure 4.18) contained 11 focus genes, including TTR, PENK, CYP19A1, WFS1, and MC4R. The top 5 canonical pathways included from 3 to 1 focus genes. The canonical pathway with the highest number of molecules (p = 4.1E-03, ratio = 0.01) was "G-Protein Coupled Receptor Signalling" with the genes MC4R, MC5R and RAS guanyl releasing (RASGRP1). The upstream analysis identified a significant association between the synthetic glucocorticoid dexamethasone and the genes CYP19A1, MC4R, PENK, TTR and fibulin 1 (FBLN) (p = 1.17E-02).

Interacting pre- and post-hatching B responsive genes: "*cumulative effect*" (category IV, Figures 4.3-IV and 4.13-IV)**.** Out of 29 pre- and post-hatching B responsive genes showing cumulative responses in the adult birds that experienced both the B-treatment protocols, 26 successfully mapped to IPA server and were all "focus" genes (Table A7a, Appendix). The significant biological functions associated with the cumulative effects induced by pre- and post-hatching B are shown in Table A8a (Appendix). The most enriched biological category was "Molecular Transport" and included 3 genes encoding solute carrier transporters (SLC6A11, SLC24A3, and SLC4A11), the gene encoding the glutamate receptor GRIP2, and the G-protein coupled receptor tachykinin receptor 1 (TACR1). A total of 2 networks were identified (scores 47 and 17) and included all the 26 focus genes. The network with the highest score included 18 focus genes and was associated with "Neurological Disease, Cell Morphology, Cell-To-Cell Signalling and Interaction" (Figure 4.19) and included SLC4A11, SLC6A11,

myosin VIIA (MYO7A), and the growth differentiation factor 10 (GDF10). There were 3 significant canonical pathways associated with GABA Receptor Signalling and Glutamate degradation ($p \leq$ 7.62E-03; ratio: 0.25-0.08) with the gene glutamate decarboxylase 1 (GAD1) and the solute carrier family 6 (SLC6A11).

4.4.8.2 Hypothalamus

Pre- and post-hatching B responsive genes (category I, Figures 4.2-I and 4.15-I). 12 out of 19 pre- and post-hatching B responsive genes mapped to IPA server. All the mapped genes except for one were "focus" genes (Table A7b, Appendix). The main biological functions included only between 1 and 4 genes and are shown in Table A8b (Appendix). The top biological function was "Behaviour" with the genes encoding the neuropeptides hormone cholecystokinin (CCK) and the Rac GTPase activating protein 1 (RACGAP1), which have been shown to be linked with emotional and anxiety-like behaviour. The most enriched biological class was "Cancer" that included again CCK and RACGAP1, plus calbidin 2 (CALB2), and the transcription regulator prospero homeobox 1 (PROX1). The upstream analysis revealed that CCK is a B-dependent gene (p = 4.87E-02). A total of 2 networks with respectively 9 and 3 focus genes (score 25 and 3, respectively) were identified. The top network was associated with "Energy Production, Nucleic Acid Metabolism and Small Molecule Biochemistry" (Figure 4.20) and included CCK, RACGAP1, PROX1, CALB2.

Pre-hatching B responsive genes (category II, Figures 4.2-II and 4.15-II). Out of 14 specifically pre-hatching B responsive genes, 12 mapped to the IPA server. All the submitted genes apart from PRDM12 were "focus" genes (Table A7b, Appendix). The significant biological functions are reported in Table A8b (Appendix). The top biological function was linked with lipid metabolism and contained 5 focus genes: glycoprotein hormones, alpha polypeptide (CGA); luteinising hormone, choriogonadotrophin receptor (LHCGR); protein kinase C, beta (PRKCB); transient receptor potential cation channel, subfamily C, member 4 (TRPC4), and hematopoietic prostaglandin D sinthase (HPGDS). The top significant biological category containing the highest number of genes was

associated with "Molecular Transport" (Table A8b, Appendix). Two networks (scores 28 and 3) were identified and overall contained respectively 10 and 1 focus genes. Among the genes incorporated in the top network (Figure 4.21) there were the gene encoding HPDGS, CGA, LHCGR, RAS, dexamethasone induced 1 (RASD1) and inositol 1, 4, 5 – trisphosphate receptor, type 3 (ITPR3). The top canonical pathway was "nNOS Signaling in neurones" (associated with nitric oxide formation) and included PRKCB and RASD1 (p = 5.24E-04, ratio = 0.038). The upstream analysis revealed a significant association between dexamethasone and the CGA, RASD1 as well as ITPR3 (p = 3.01E-02).

Post-hatching B responsive genes (category III, Figures 4.2-III and 4.15-III). Out of 88 post-hatching B responsive genes, 72 mapped to the IPA server. Of these 72 genes, 66 were "focus" genes (Table A7b, Appendix). The significant biological functions are reported in Table A8b (Appendix). The top significant biological function was "Neurological Disease" with 27 genes and included 3 types of serotonin receptors (HTR1D, HTR2C, and HTR3A); corticotrophin releasing hormone receptor 2 (CRHR2); adenosine A1 receptor (ADORA1); cytochrome P450 family 27, subfamily A, polypeptide 1 (CYP27A1); somatostatin receptor 5 (SSTR5). Not surprisingly, the upstream analysis revealed a dependency between SSTR5 and CYP27A1 and the synthetic stress hormone dexamethasone (p = 4.93E-02). Some of the above mentioned genes were also linked with several behavioural traits, such as anxiety, learning and cognition (ADORA1, CRHR2, and HTR2C). A total of 7 networks were identified and their scores ranged from 39 to 2 and all together included 63 out of 66 focus genes. The first top network (Figure 4.22) incorporated 18 focus genes (all up-regulated) and was associated with "Neurological Disease, Psychological Disorders, Cell Signalling". This network included the genes encoding the 3 serotonin receptors (HTR1D, HTR2C, and HTR3A), ADORA1, SSTR5 as well as CRHR2. The top canonical pathway was "Serotonin Receptor Signalling" (p = 2.38E-04, ratio = 0.067) with all the 3 serotonin receptors mentioned above.

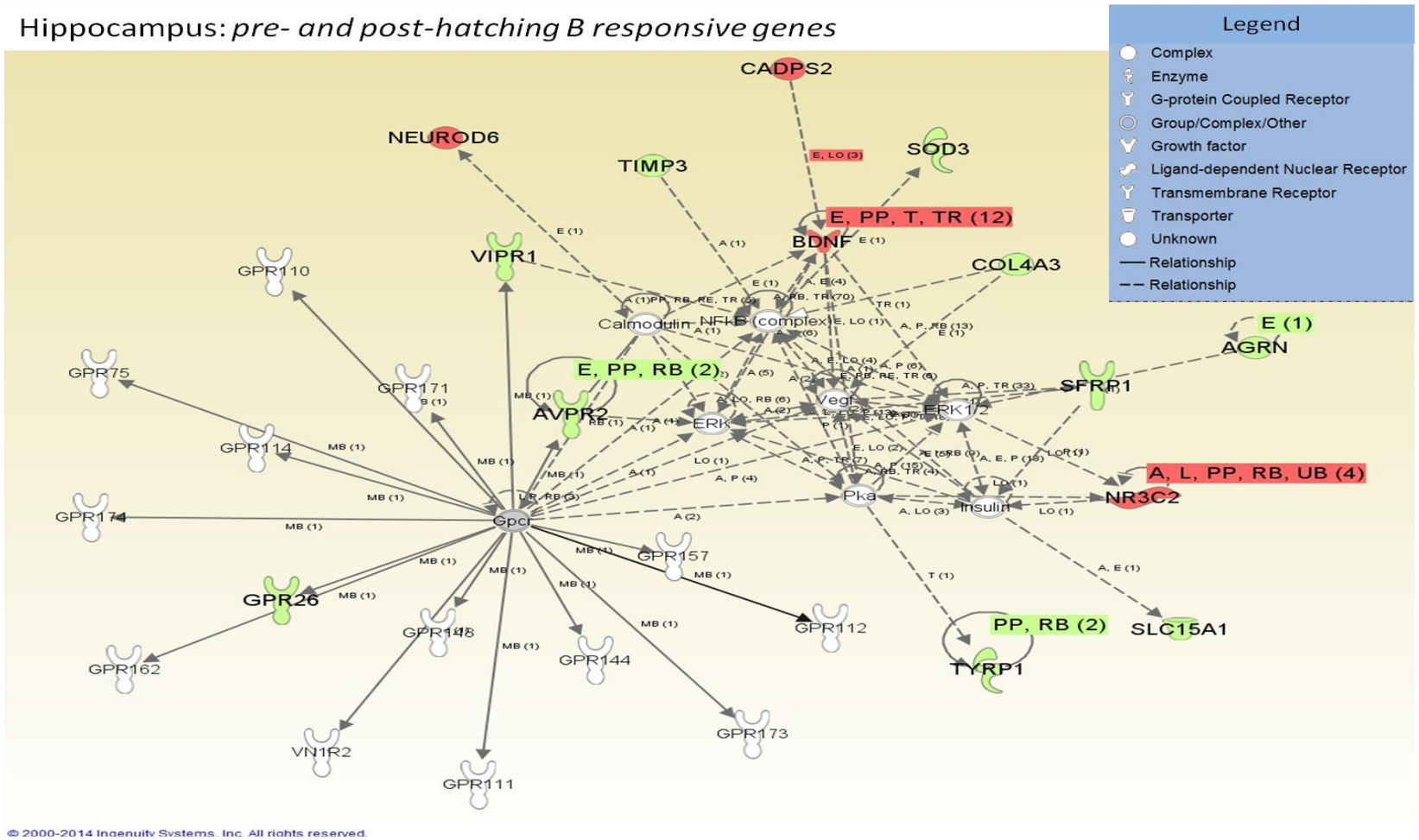

Figure 4.17 First top significant network generated by Ingenuity Pathway Analysis showing the down-regulated genes (green) and up-regulated genes (red) in the hippocampus (score: 30) that were altered by both pre- and post-hatching corticosterone (B) exposure. The network is displayed with nodes (i.e. genes) and edges (i.e. biological interactions among nodes); in white, the genes not user-specific added into the network due to interactions with the submitted genes. Solid lines connecting the genes indicate direct interactions between the nodes and dashed lines implied indirect interactions.

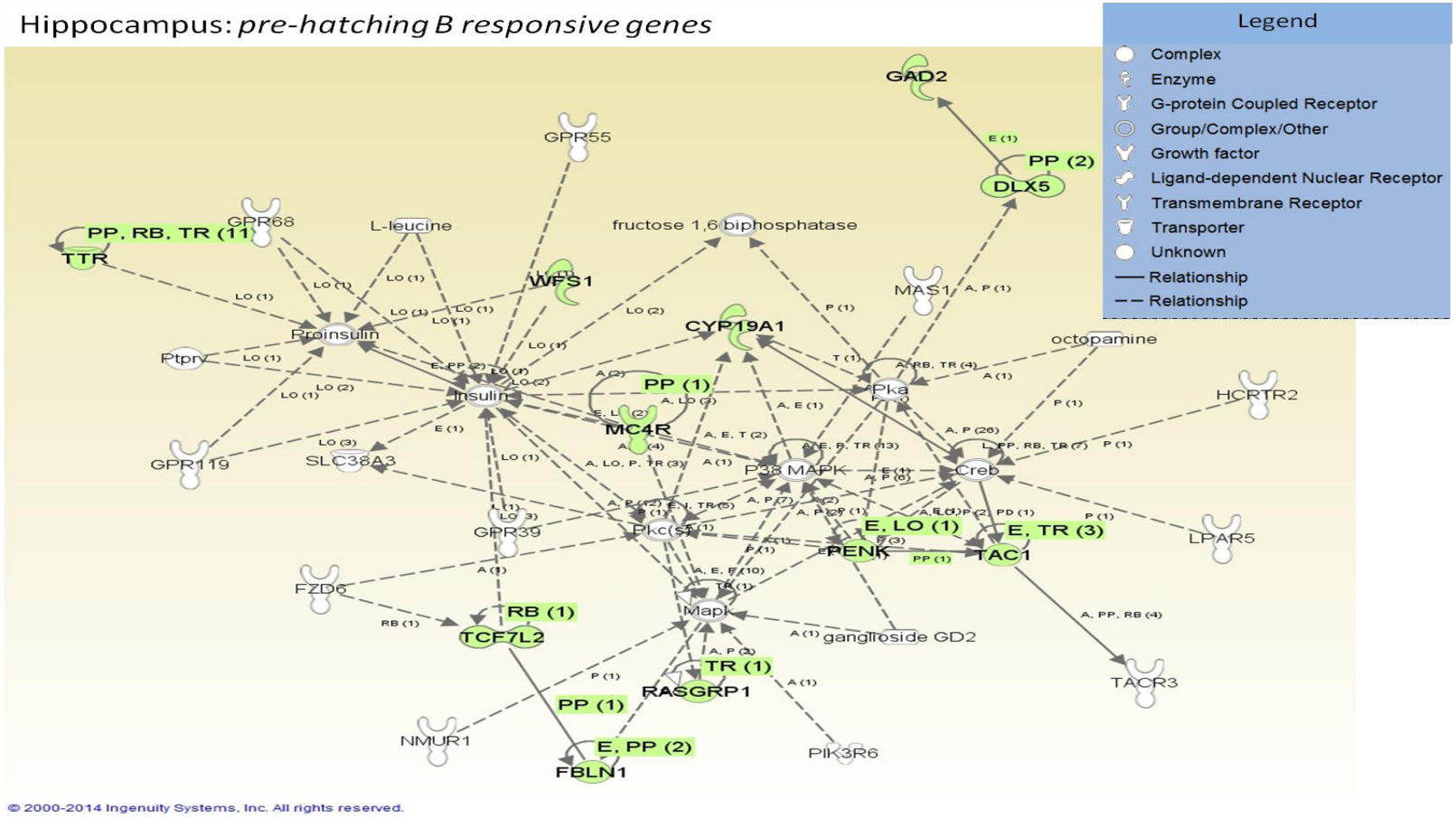

Figure 4.18 First top significant network generated by Ingenuity Pathway Analysis showing the down-regulated genes (green) and up-regulated genes (red) in the hippocampus (score: 27) that were specifically altered by pre-hatching corticosterone (B) exposure. The network is displayed with nodes (i.e. genes) and edges (i.e. biological interactions among nodes); in white, the genes not user-specific added into the network due to interactions with the submitted genes. Solid lines connecting the genes indicate direct interactions between the nodes and dashed lines implied indirect interactions.

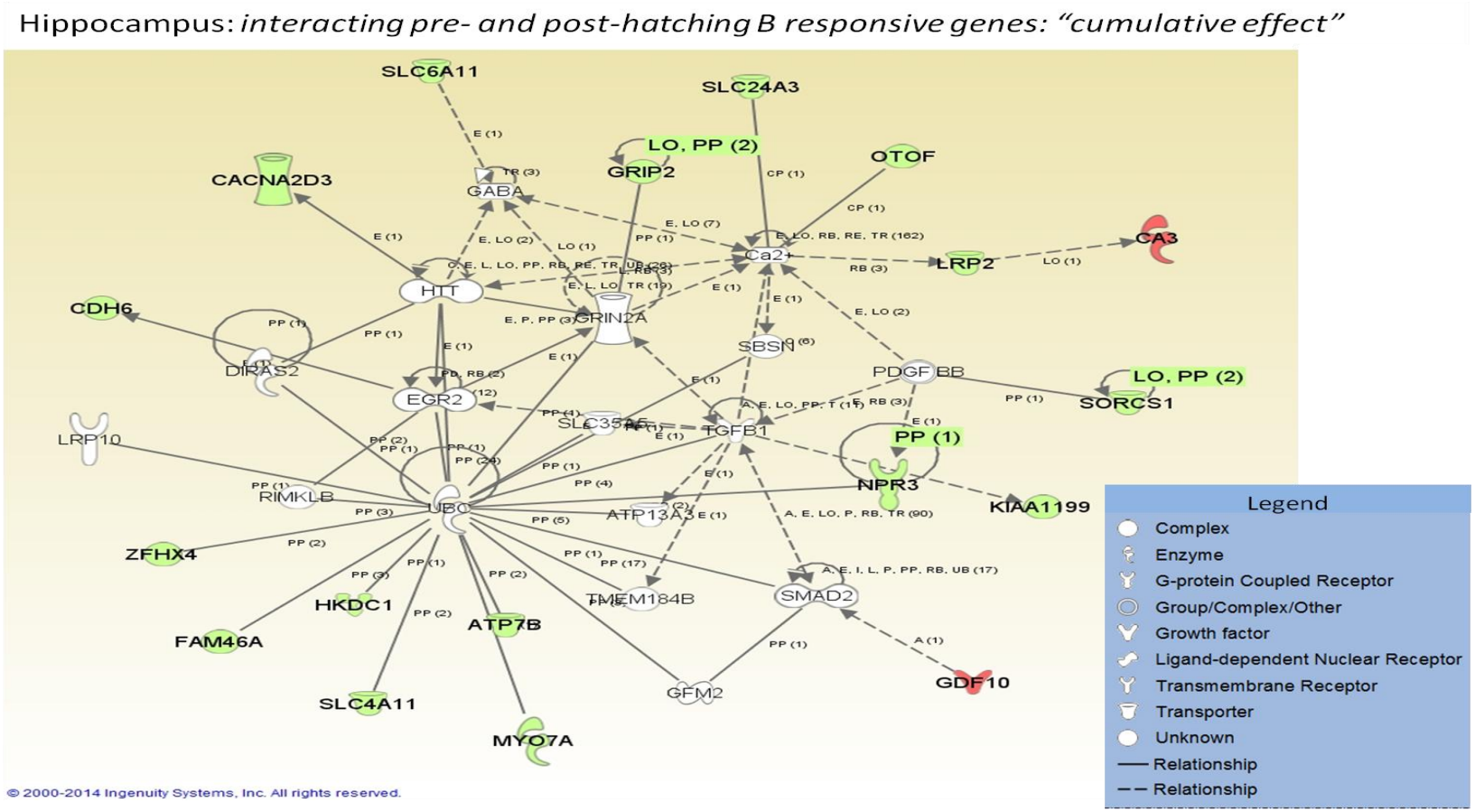

Figure 4.19 First top significant network generated by Ingenuity Pathway Analysis showing the down-regulated genes (green) and up-regulated genes (red) in the hippocampus (score: 47) that induced cumulative effects in the birds that were exposed to both pre- and post-hatching corticosterone (B). The network is displayed with nodes (i.e. genes) and edges (i.e. biological interactions among nodes); in white, the genes not user-specific added into the network due to interactions with the submitted genes. Solid lines connecting the genes indicate direct interactions between the nodes and dashed lines implied indirect interactions.

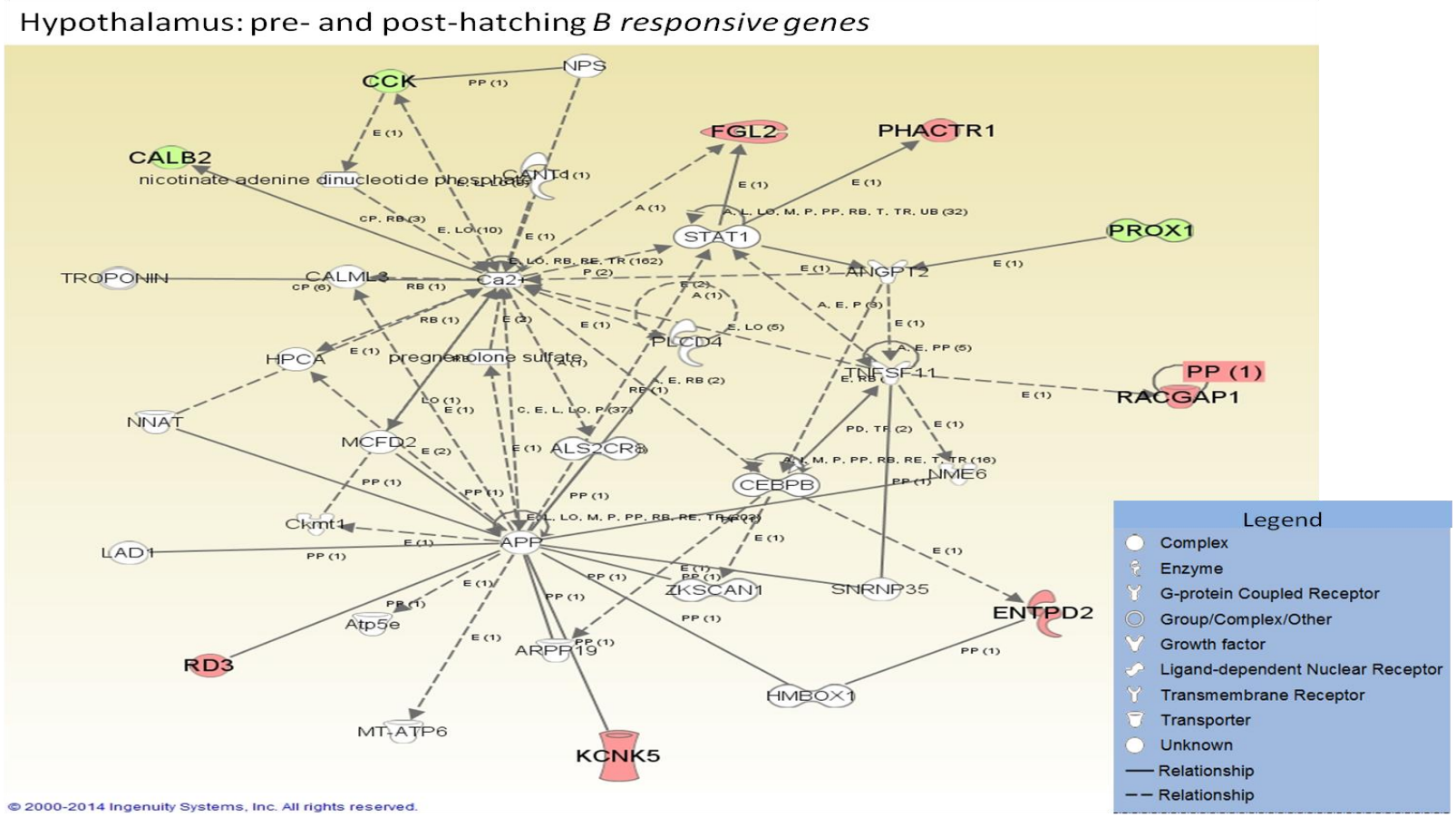

Figure 4.20 First top significant network generated by Ingenuity Pathway Analysis showing the down-regulated genes (green) and up-regulated genes (red) in the hypothalamus (score: 25) that were altered by both pre- and post-hatching corticosterone (B) exposure. The network is displayed with nodes (i.e. genes) and edges (i.e. biological interactions among nodes); in white, the genes not user-specific added into the network due to interactions with the submitted genes. Solid lines connecting the genes indicate direct interactions between the nodes and dashed lines implied indirect interactions.

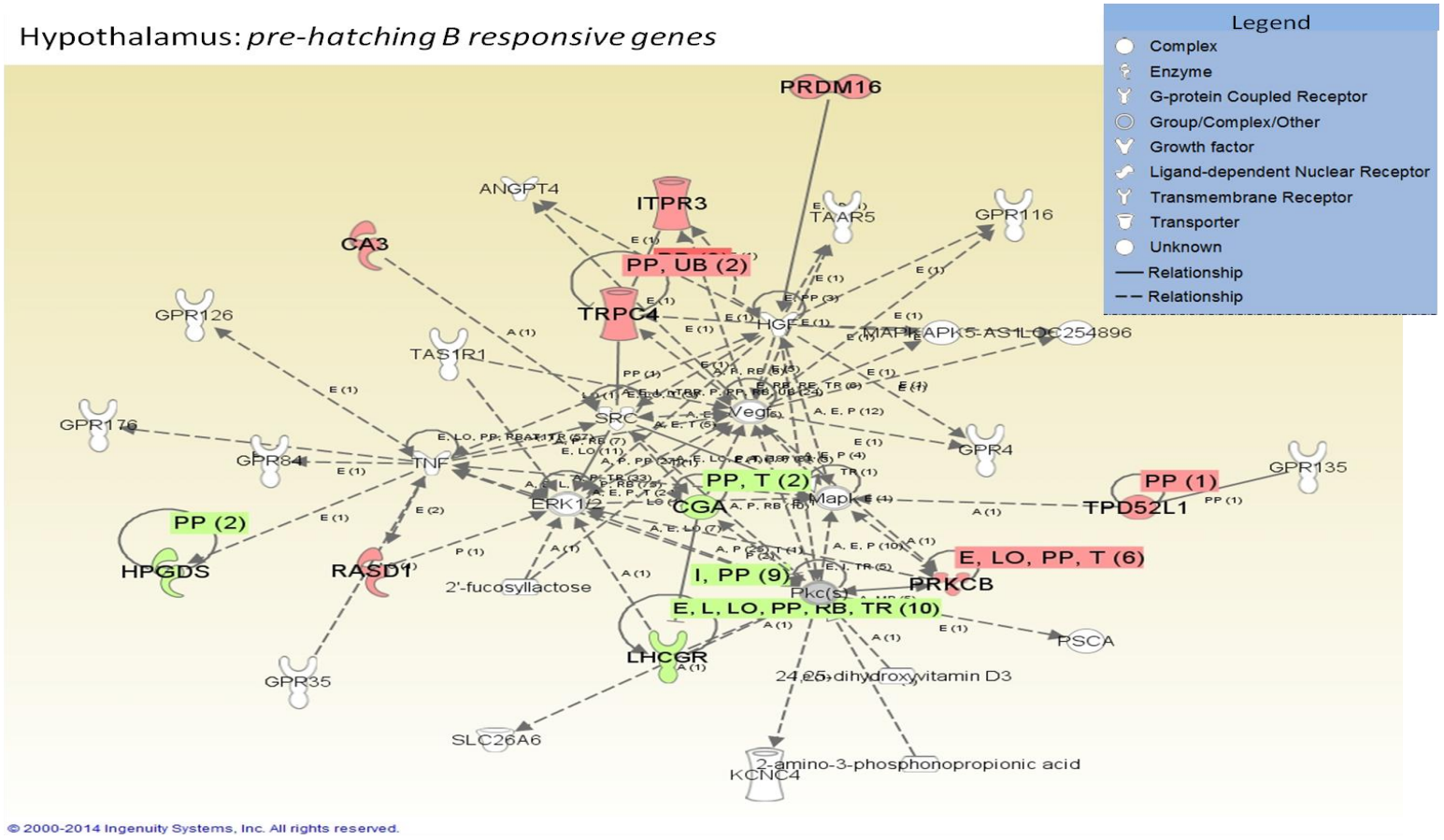

Figure 4.21 First top significant network generated by Ingenuity Pathway Analysis showing the down-regulated genes (green) and up-regulated genes (red) in the hypothalamus (score: 28) that were specifically altered by pre-hatching corticosterone (B) exposure. The network is displayed with nodes (i.e. genes) and edges (i.e. biological interactions among nodes); in white, the genes not user-specific added into the network due to interactions with the submitted genes. Solid lines connecting the genes indicate direct interactions between the nodes and dashed lines implied indirect interactions.

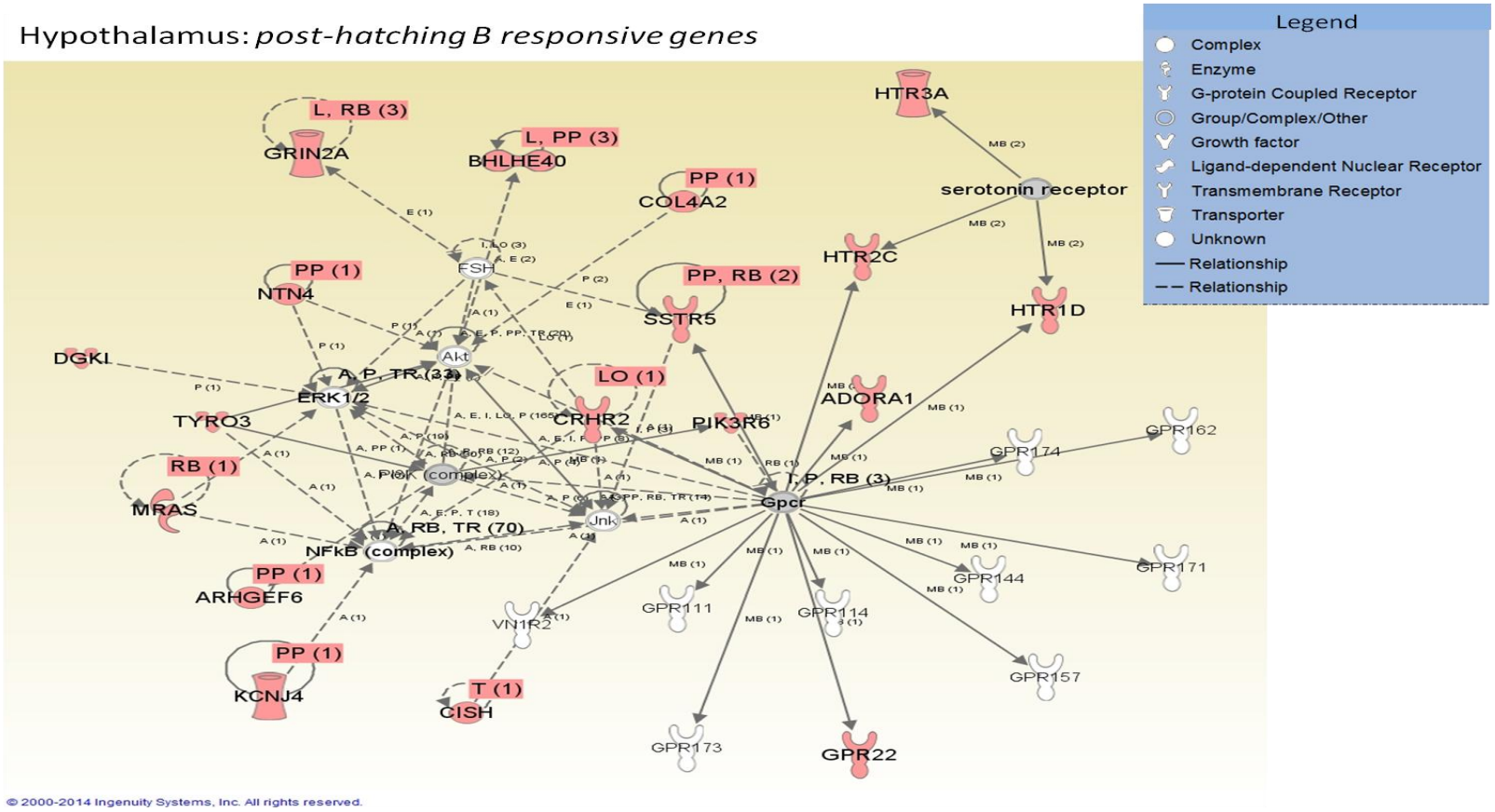

Figure 4.22 First top significant network generated by Ingenuity Pathway Analysis showing the down-regulated genes (green) and up-regulated genes (red) in the hypothalamus (score: 39) that were specifically altered by post-hatching corticosterone (B) exposure. The network is displayed with nodes (i.e. genes) and edges (i.e. biological interactions among nodes); in white, the genes not user-specific added into the network due to interactions with the submitted genes. Solid lines connecting the genes indicate direct interactions between the nodes and dashed lines implied indirect interactions.

4.4.9 Validation

4.4.9.1 Microarrays

4.4.9.1.1. PCA

The PCA explained 41.7% of the overall variation in global hippocampal gene expression. The PCA mapping showed a good clustering across the replicates within each treatment group, although variation was high both in the CC and BB groups (Figure 4.23). Consistently with the RNA-seq data, there was a clear separation between the CC and the BC birds along the horizontal PC1 (Figure 4.23).

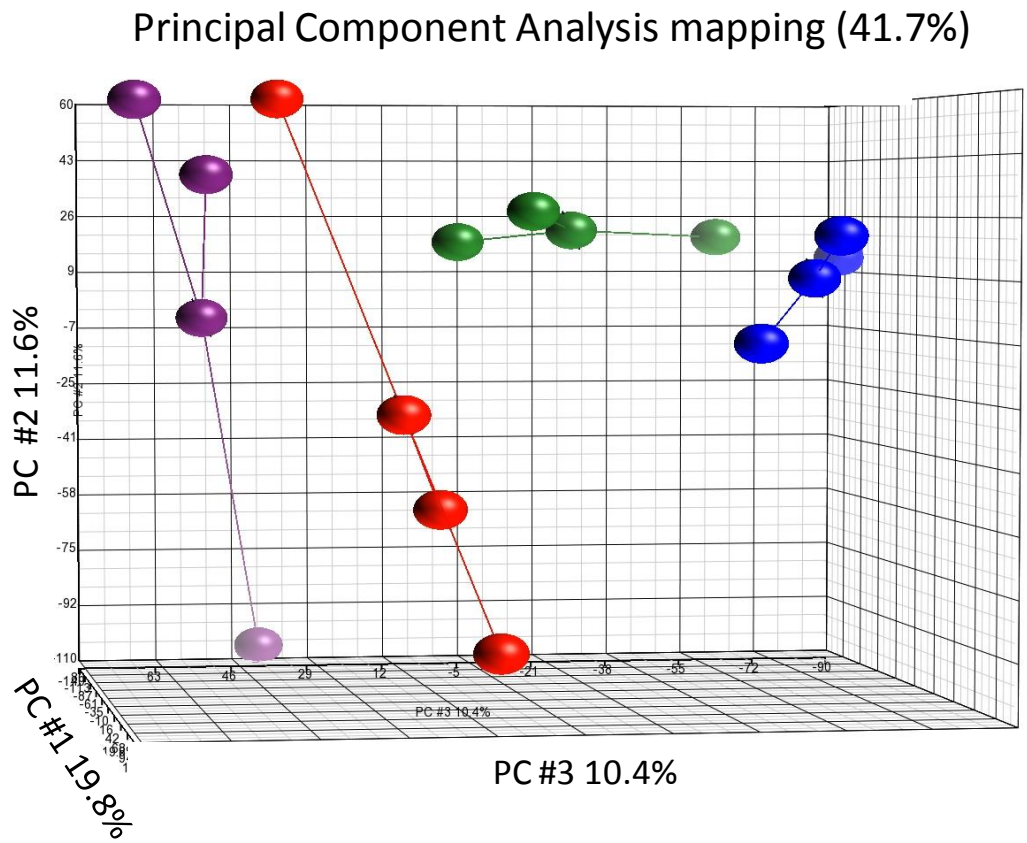

Figure 4.23 Principal Component Analysis (PCA) mapping of the hippocampal microarray samples (n = 12) using the normalised expression abundance values of the annotated (with an Ensembl Identifier and/or gene name) probesets (n = 26522). Data are clustered by treatment group (CC in purple, BC in blue, CB in green or BB in red) using the centroid function. PC1 = first component, explaining 19.8% of the overall variation; PC2 = second component, explaining 11.6%, and PC3 explaining 10.4% of the variation across annotated probesets.

4.4.9.1.2 Two-way ANOVA

There were no effects of pre-hatching or post-hatching treatment as main factor (q > 0.2 in both). There were no interaction effects between the pre- and post-hatching treatments (interactions: pre-hatching B X post-hatching B, pre-hatching B X pre-hatching C, post-hatching B X post-hatching C, all q > 0.2). The post-hoc comparison found no differentially expressed genes, except two genes (scaffold attachment factor B2, SAFB2; tektin 3, TEKT3; q < 0.05 in both) in the contrast BB *vs* CB.

4.4.9.1.3 RankProducts

The number of the differentially expressed genes across the pair-wise comparisons is shown in Table 4.7.

Table 4.7 Number of significant genes (FDR ≤ 0.10) that were up- or down- regulated across the pair-wise comparisons in the hippocampal samples.

Contrast: (2^{nd} class vs 1^{st} class)	Up-regulated genes under 2^{nd} class	Down-regulated genes under 2^{nd} class
BC vs CC	47	100
CB vs CC	22	35
BB vs CC	18	89
CB vs BC	52	52
BB vs BC	65	48
BB vs CB	89	60

As observed in the RNA-seq analysis, the largest number of differentially expressed genes was skewed towards a down-regulation in the pre-hatching B-treated birds (BC and BB) when compared with the controls in the contrast BC *vs* CC and BB *vs* CC. In fact, the fold changes in the contrast BC *vs* CC for the down-regulated genes ranged from -18.33 to -2.48, and from -26.64 to -2.39 in the contrast BB *vs* CC; whilst the fold changes for the significantly up-regulated genes ranged from 12.56 to 2.49 in BC *vs* CC comparison and from 8.16 to 2.82 in

BB *vs* CC. Genes of relevant interest that were repressed in the birds that experienced exposure to B *in ovo* alone when compared to the adult controls where vasotocin-neurophysin VT (FDR = 0.04); transthyretin (FDR = 0.04); superoxide dismutase 3, extracellular (FDR = 0.05) and glutathione S-transferase alpha 3 (FDR = 0.04), both regulating oxidation processes; and two guanine nucleotide binding G-protein (gamma 11 and gamma 13; FDR = 0.07 for both). Interestingly, transthyretin; superoxide dismutase 3, extracellular, and guanine nucleotide binding G-protein 11 were also significantly repressed in the pre- and post-hatching B-treated birds when compared to the adult controls (0.005 <FRD<0.04). Also, down-regulated in the BB birds when compared with CC birds where the genes proenkephalin (FDR = 0.03) and somatostatin receptor II (FDR = 0.06). Although the effects of post-hatching B appeared much less pronounced than those induced by pre-hatching B, it is important to remark that vasotocin-neurophysin VT, transthyretin and proenkephalin were in contrast up-regulated (0.01 < FDR < 0.09) in the CB birds compared with the BB birds.

4.4.9.1.4 Comparison of absolute gene expression values from RNA-seq and Microarrays

There was a high dispersion in the scatter plots across the entire range of gene expression values measured by RNA-seq and Microarrays, although the genes expressed at higher abundance where better correlated than those expressed at lower abundance in at least one of the platform (Figure 4.24) Despite the correlation between the RNA-seq $\log_2$ counts and the Microarray $\log_2$ intensities was highly statistically significant (p < 2.2E-16 for all), the Spearman's correlation coefficient (rho) was relatively low (mean ± SEM: 0.58 ± 0.17), ranging from 0.57 to 0.60.

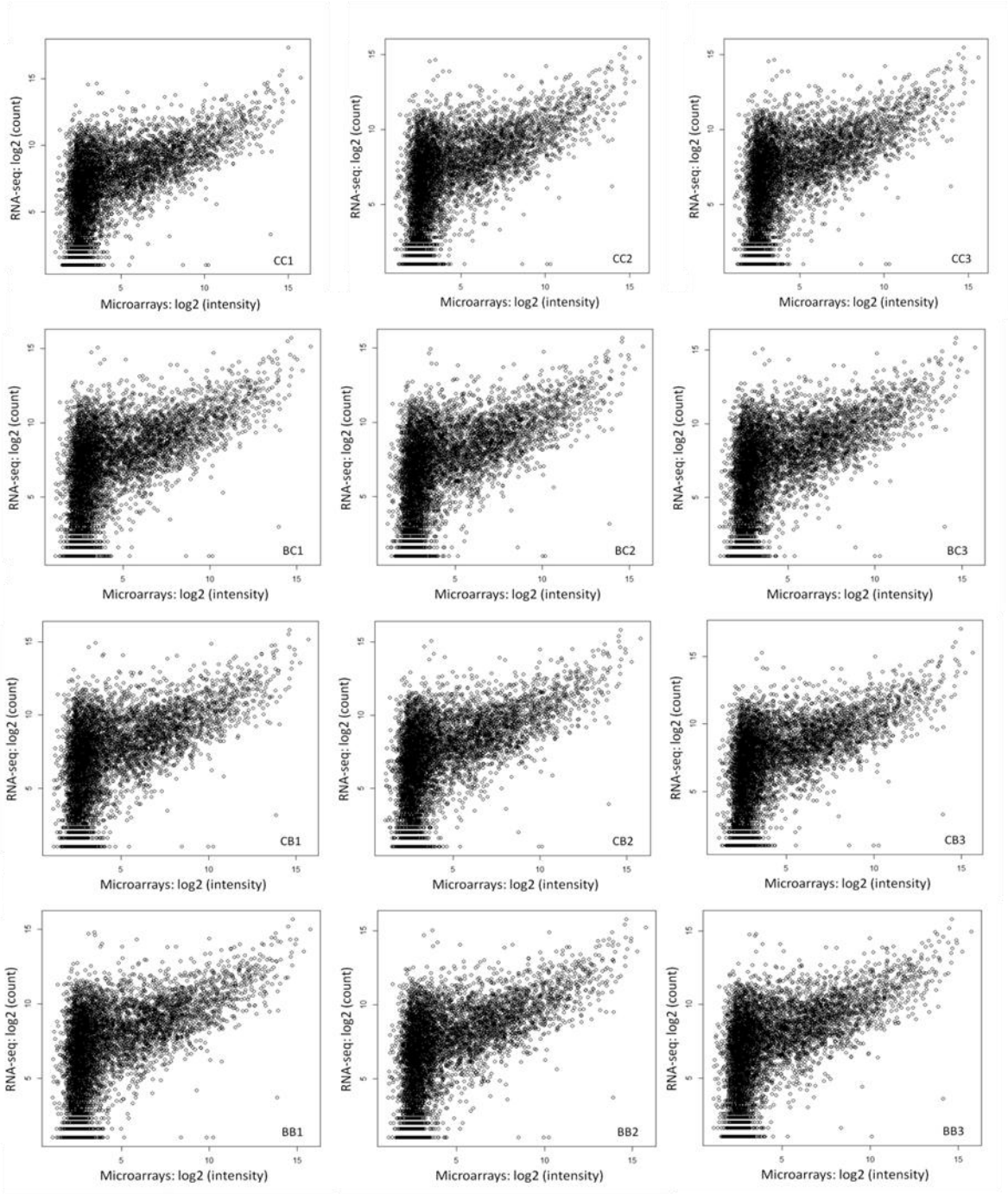

Figure 4.24 Inter-platform expression correlation plots between the Microarrays data and RNA-sequencing data based on 7172 common non-redundant Ensembl transcripts. Each panel shows RNA-sequencing $\log_2$ counts compared to Microarray $\log_2$ intensity values derived from the same RNA hippocampal samples. In each graph is indicated the treatment group (CC, BC, CB or BB) with the numbers (1, 2, 3) representing the biological replicate in each treatment group.

4.4.9.1.5 Comparison of the differentially expressed genes between RNA-seq and Microarrays

The number of genes from both RNA-seq and Microarrays with a FDR cut-off of 0.20 and the number of shared genes between the two platforms are shown in the Figures 4.25-4.26; the Ensembl identifiers, annotation of these genes and fold-changes in both the platforms is reported in Table A9 (Appendix).The percentage (%) of overlapping gens between RNA-seq and Microarray data ranged from 44.16% to 0 (mean ± SEM: 15.58% ± 4.50%), indicating a poor overall concordance between the two platforms. The maximal concordance was found in the down-regulated genes in the adult birds that had experienced exposure to B *in ovo* in comparison with the adult control birds (BC *vs* CC: 44.16% of shared genes; BB *vs* CC: 34.44% of shared genes); interestingly, 18 genes were consistently down-regulated in both these two comparisons (Table A10, Appendix). Among the relevant top down-regulated genes there were transthyretin; superoxide dismutase 3, extracellular and glutathione S-transferase alpha 3.

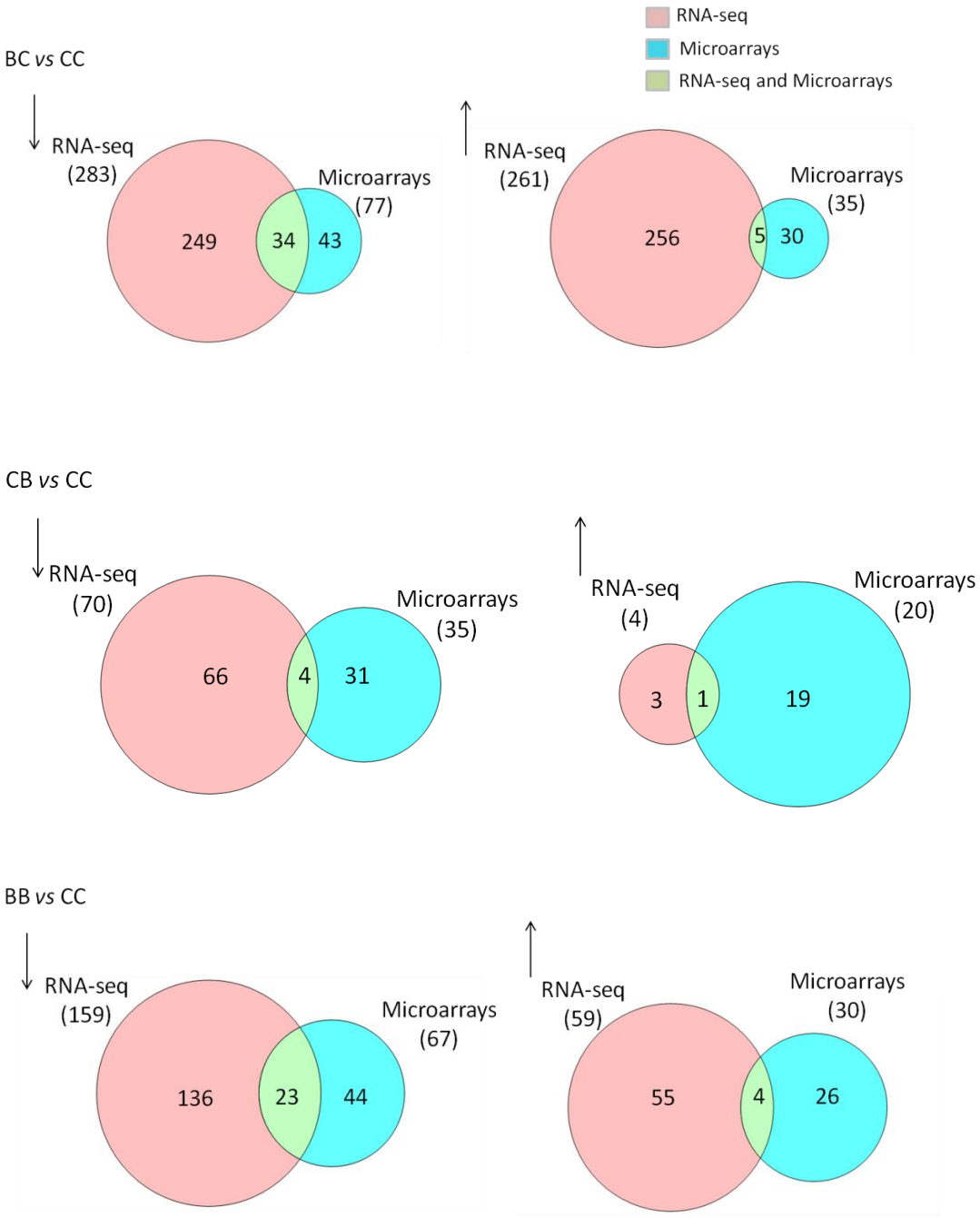

Figure 4.25 Proportional Venn Diagrams showing all the number of genes in common between RNA-seq and Microarrays at FDR ≤ 0.20 in the contrasts BC *vs* CC, CB *vs* CC and BB *vs* CC in the hippocampal samples. Comparisons were performed separately for down- and up-regulated genes (as indicated by the arrows); gene expression directional changes refer to the 2nd class *vs* 1st class.

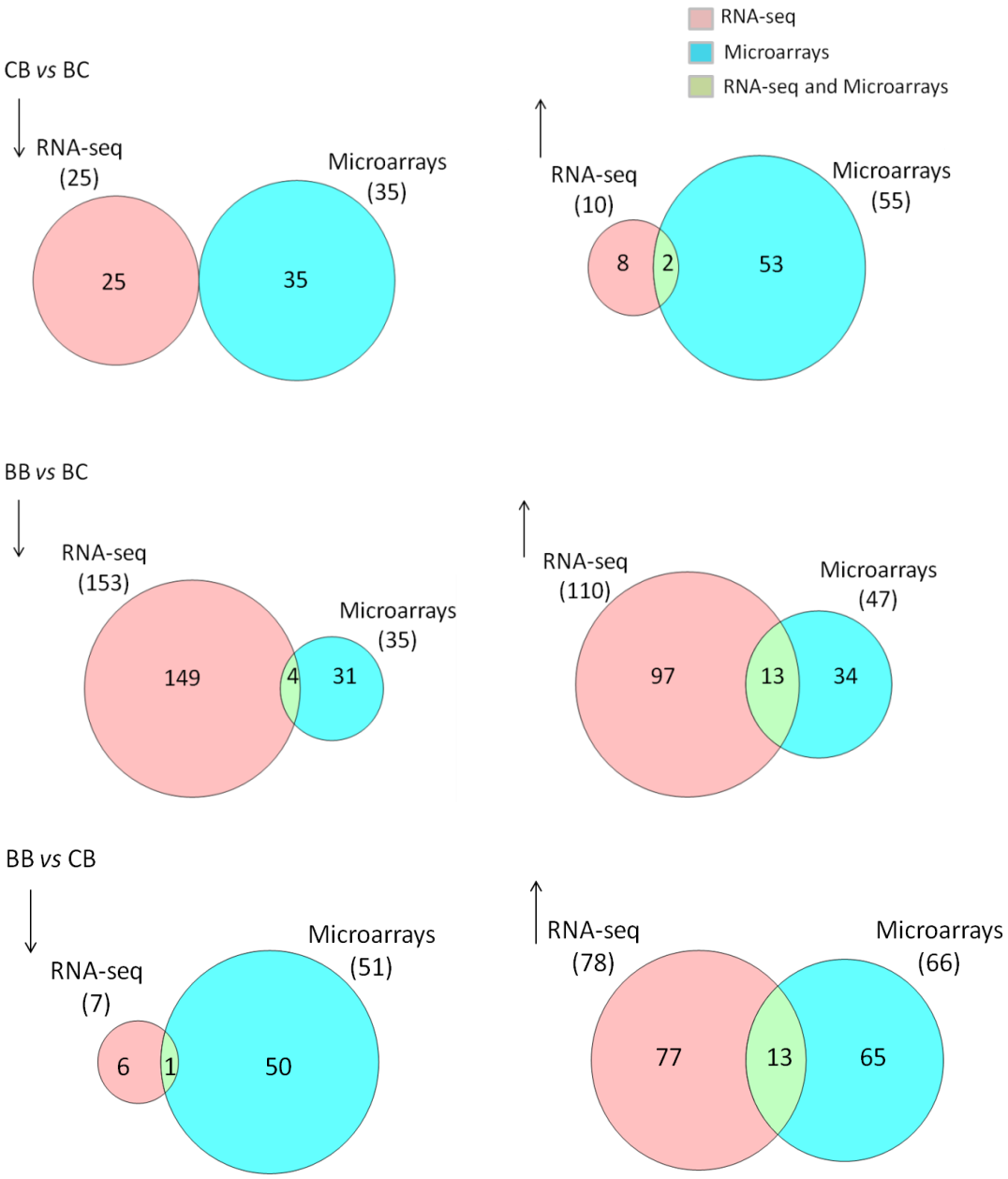

Figure 4.26 Proportional Venn Diagrams showing all the number of genes in common between RNA-seq and Microarrays at FDR ≤ 0.20 in the contrasts CB *vs* BC, BB *vs* BC and BB *vs* CB in the hippocampal samples. Comparisons were performed separately for down- and up-regulated genes (as indicated by the arrows); gene expression directional changes refer to the 2nd class *vs* 1st class.

4.4.9.2 qPCR

The comparative data showing the expression patterns obtained by using RNA-seq and Microarrays for the genes AVP, TTR, SOD3, GSTA3 and GNG11 are in good agreement with those obtained by using qPCR (Figures 4.27-4.29); in fact, almost all genes showed concordant directional fold changes across the three

techniques (Figures 4.30-4.31). The few discrepancies observed for SOD3, GSTA3, and GNG11 regarded expression differences of low intensity (approximately absolute fold change of 2).

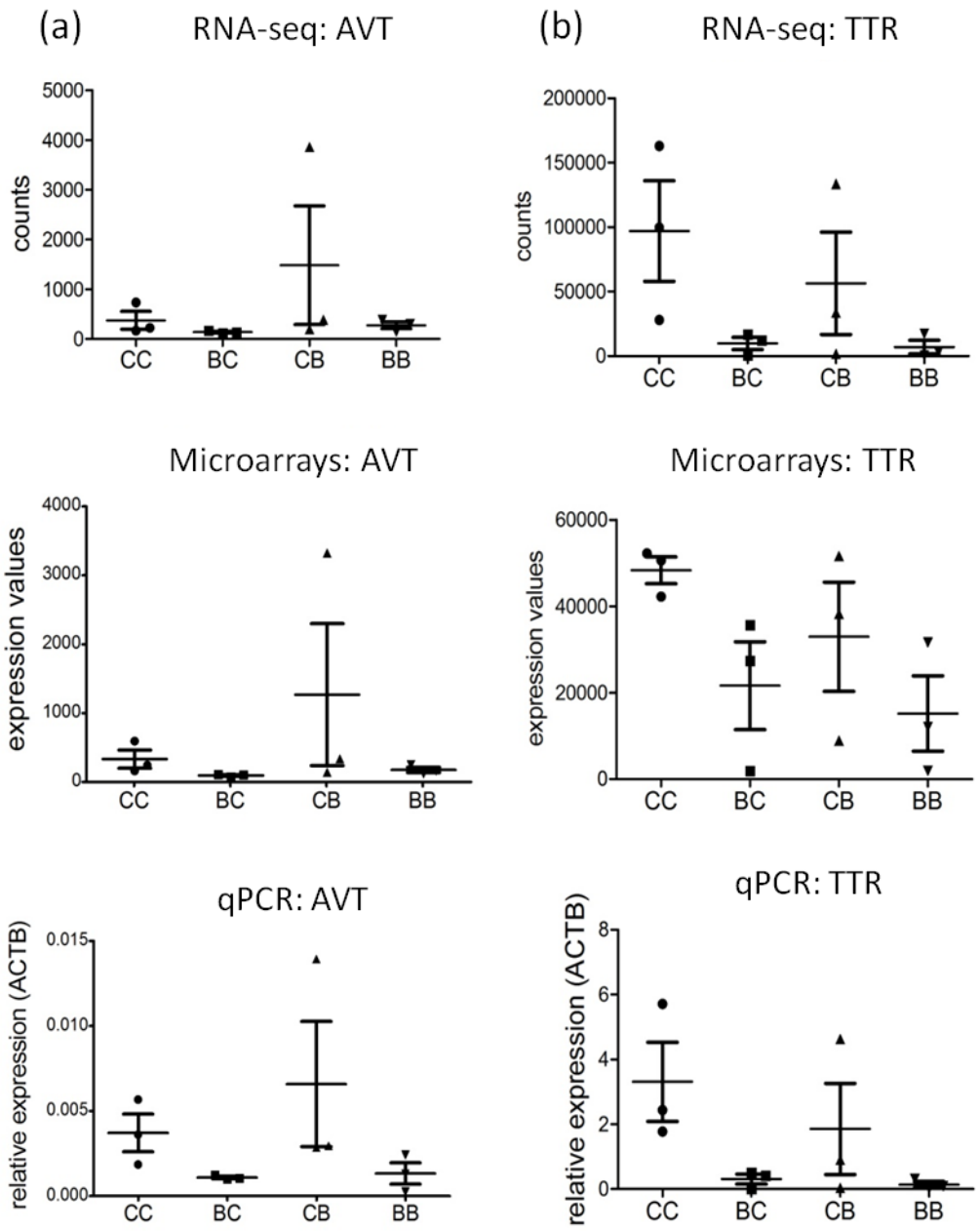

Figure 4.27 Absolute expression values for the genes (a) AVT and (b) TTR from RNA-seq, Microarrays and qPCR. RNA-seq counts were obtained using HT-Seq; Microarrays expression intensities were normalised using the GC-content by the Robust Multichip Average; qPCR expression values were normalised using the comparative threshold method relatively to β-actin (ACTB).

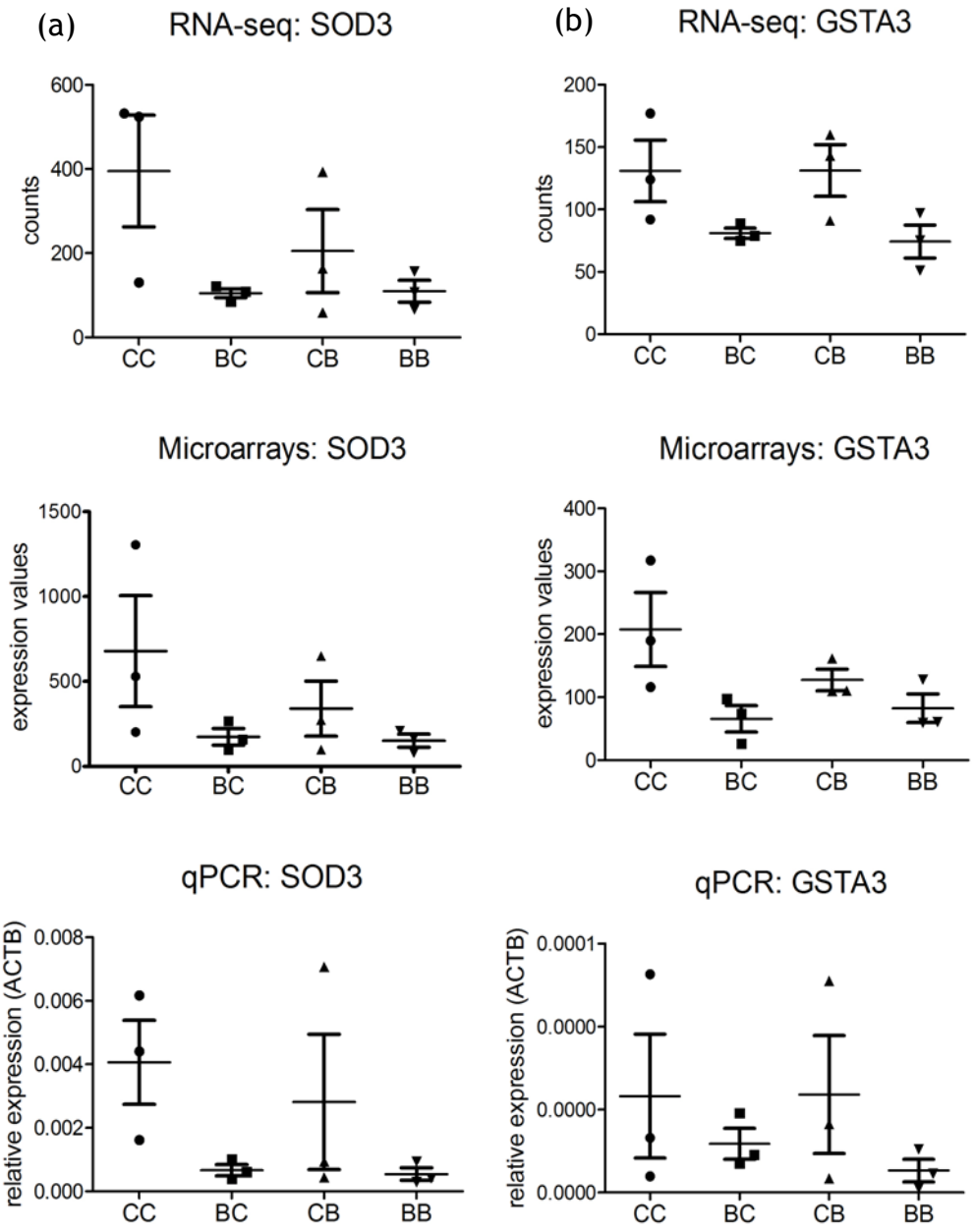

Figure 4.28 Absolute expression values for the genes (a) SOD3 and (b) GSTA3 from RNA-seq, Microarrays and qPCR. RNA-seq counts were obtained using HT-Seq; Microarrays expression intensities were normalised using the GC-content by the Robust Multichip Average; qPCR expression values were normalised using the comparative threshold method relatively to β-actin (ACTB).

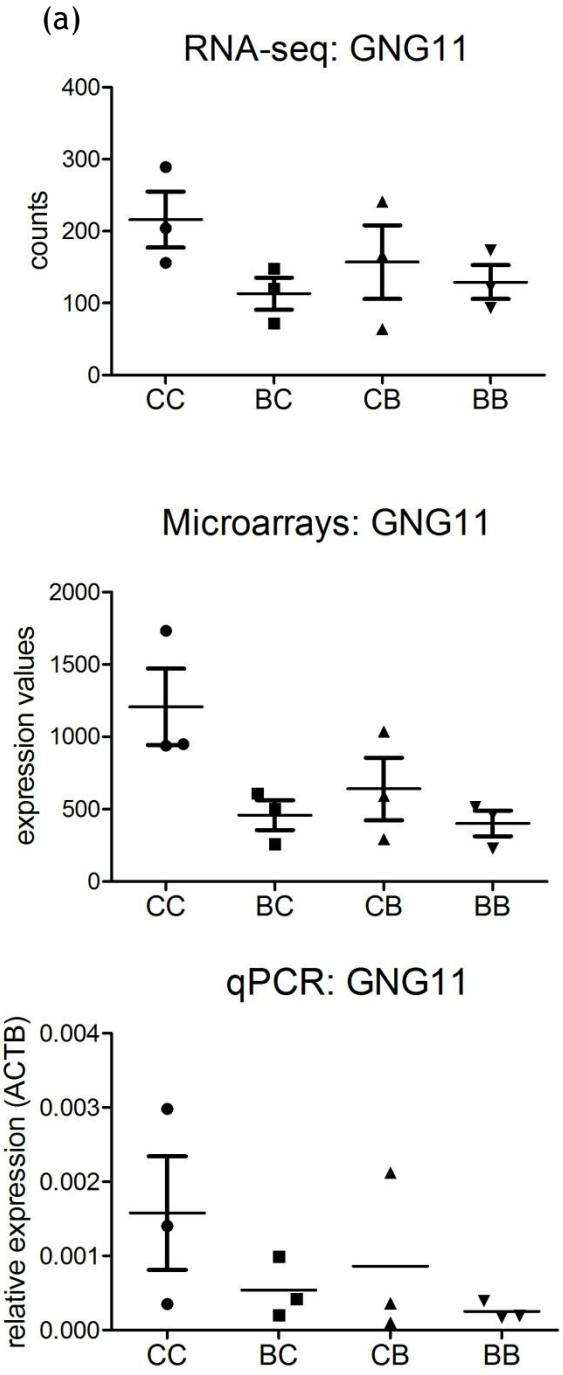

Figure 4.29 Absolute expression values for the gene GNG11 from RNA-seq, Microarrays and qPCR. RNA-seq counts were obtained using HT-Seq; Microarrays expression intensities were normalised using the GC-content by the Robust Multichip Average; qPCR expression values were normalised using the comparative threshold method relatively to β-actin (ACTB).

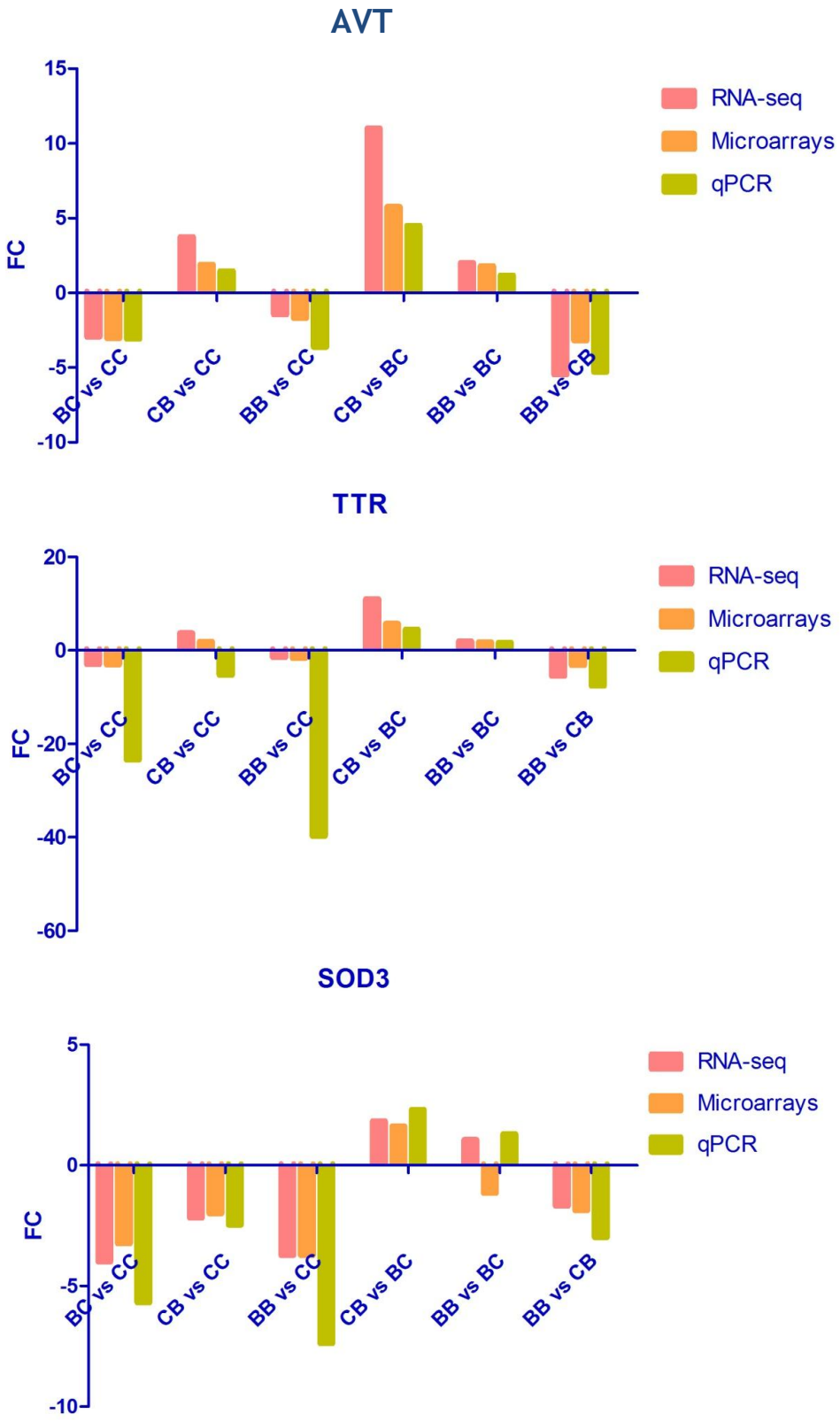

Figure 4.30 Fold changes for AVT, TTR, and SOD3 derived on the basis of samples processed using RNA-seq, Microarrays and qPCR across the 6 pair-wise comparisons. Plotted values represent expression averages from the 3 biological replicates.

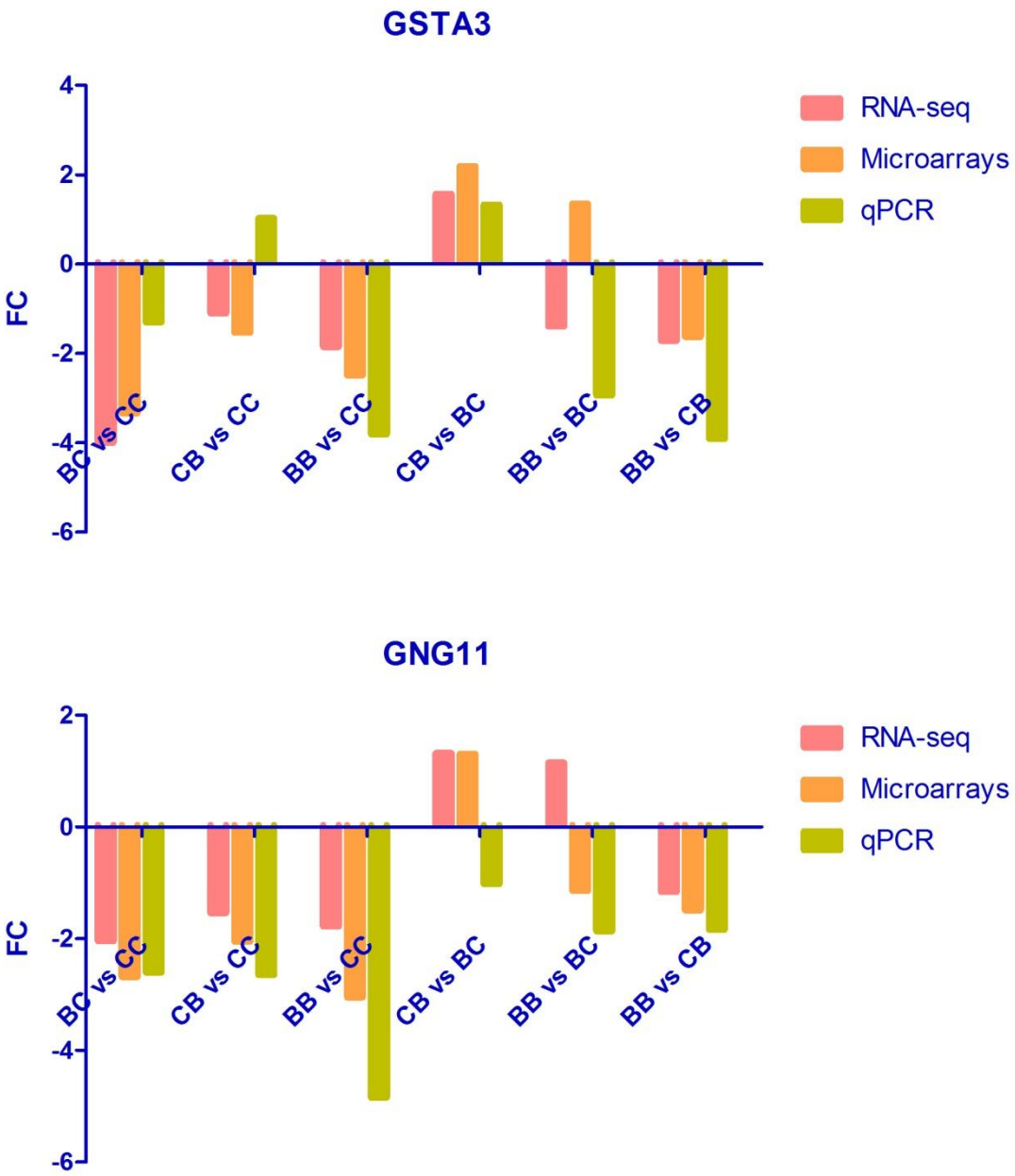

Figure 4.31 Fold changes for GSTA3 and GNG11 derived on the basis of samples processed using RNA-seq, Microarrays and qPCR across the 6 pair-wise comparisons. Plotted values represent expression averages from the 3 biological replicates.

4.5 Discussion

The findings presented in this study suggested that the Japanese quail exposed to exogenous B as embryos and/or juveniles showed distinct tissue-specific modifications in global gene expression patterns in their hippocampi and hypothalami when adults. The genes' dynamic responses to pre- and post-hatching B were overall weak, but discernible and involved well characterised

key candidate genes in the regulation of the HPA system, such as mineralocorticoid receptor (MR), vasotocin-neurophysin VT (AVT; homolog of arginine vasopressin in mammals) and its receptor AVTR2, somatostatin and serotonin receptors, as well as in the priming actions of early life experiences, such as brain-derived neurotrophic factor (BDNF) and Ca++ - dependent secretion activator 2 (CADPS2). This study also contributed to novel information regarding the potential regulatory mechanisms driving the long-term effects of early life stress, likely to be linked with oxidative stress. These data, to the best of my knowledge, represent the first attempt that had experimentally quantified the long-term potential cumulative and opposing gene expression responses resulting from the combined exposure to pre- and post-hatching B.

The overall weak gene expression changes observed in this experiment are in line with the few previous studies that have examined the impact of variations in early life environmental experiences on the brain transcriptome signature over the long-term (Weaver *et al.*, 2006; Lindqvist *et al.*, 2007; Nätt *et al.*, 2009; Goerlich *et al.*, 2012). However, in this specific experimental context, the low magnitude of the treatment effect may also be the consequence of additional complicating factors that may have decreased both sensitivity and accuracy of the RNA-seq differential expression analysis, primarily the background noise due to the high variation across the limited number of biological replicates and the lack of the quail sequence genome. Despite the efforts made in order to ascertain quality, comparability and reliability of the RNA-seq data by undertaking different and novel statistical approaches as well as to validating the results using other technologies, I am unable to completely exclude the possibility that other gene expression pattern changes associated with the long-term effects of glucocorticoid exposure may be revealed by a more detailed analysis, for instance by *de-novo* assembly of the quail transcriptome. For future studies, other techniques other than genome-wide approaches, such as *in situ* hybridisation and immunohistochemistry, will also be suitable methods for further validating the biological reliability of the candidate genes reported in this study.

4.5.1 The long-term gene expression dynamic responses to B exposure during pre- and post-hatching development in the hippocampus and hypothalamus

4.5.1.1 Pre- and post-hatching B sensitive genes

The results clearly showed that physiological overexposure to B, regardless of the developmental stage, induced consistent gene expression alterations across the B-exposed phenotypes relative to the controls in adulthood. These changes were highly specific within tissues and stronger in the hippocampus than in the hypothalamus. Of particular interest were the effects observed in the hippocampus in which the expression signals of classic genetic markers of early life stress, such as MR (gene NRC32), BDNF and CADPS2, were altered and clustered together in the top significant network (Section 4.4.8.1, Figure 4.17). The functional analysis confirmed the known association of MR with B, but it also showed specific co-regulation of MR with the other two B-dependent genes, BDNF and SFRP1, via the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, which mediates cell proliferation and apoptosis (Mebratu and Tesfaigzi, 2009). The over-expression of BDNF in the B-treated birds relative to the controls was directly associated with CADPS2, which was also over-expressed in these birds. The mechanism of actions involved in the promotion of hippocampal BDNF expression in response to increases of CADPS2 has been recently elucidated in the rat model (Shinoda *et al.*, 2011). Detailed mammalian literature indicates that BDNF is a key regulator of brain development and neuronal plasticity, especially in the hippocampus where it is highly active and expressed (Ernfors *et al.*, 1991). Importantly, BDNF mRNA expression in the brain is strongly influenced by early life stressful experiences (reviewed by Cirulli *et al.*, 2009) and recent studies in birds have shown similar significant associations (Lindqvist *et al* 2007; Chaudhury and Wadhwa, 2009). Hippocampal BDNF expression in rats is altered by both pre-natal stress protocol (i.e. maternal restraint) and post-natal stressful manipulations, such as maternal separation (Roceri *et al.*, 2002; Lippmann *et al.,* 2007; Zuena *et al.,* 2008). However, the dynamics of the directional changes induced by these early life stressful protocols on BDNF expression vary across studies, probably because of differing time points, duration and intensity of the stressor employed and interactions

with other factors, such as sex (reviewed by Gomez-Pinilla and Vaynman, 2005; Cirulli *et al.,* 2009;). It becomes therefore difficult to draw a functional interpretation of the over-expression of BDNF observed in this experiment. Nevertheless, several studies in adult individuals have shown that chronic B treatment reduces mRNA BDNF expression in the hippocampus over the short-period (review by Schaaf *et al.*, 2000). Decreased BDNF levels are thought to affect hippocampus-related learning and anxiety-like behaviours (Duman and Monteggia, 2006; Martinowich *et al.*, 2007). Hence, in light of these studies, I would speculate that the over-expression of hippocampal BDNF in the B-exposed quail might be an adaptive mechanism activated to buffer potential hippocampal learning impairments or increased anxiety-related traits. Such potential effects in the B-treated quail might also have been mediated by the simultaneous up-regulation of hippocampal MR compared to the control quail. In fact, down-regulation of MR has been associated with impaired spatial memory abilities in zebra finch lines selected for exaggerated B stress responses (Hodgson *et al.*, 2007). Future studies are needed to clarify the biological validity of this hypothesis. For example, a fed-baited eight-arm radial arm maze, modified version of an eight-arm radial maze test validated in the Japanese quail (Suhr *et al*. 2010), could be a suitable behavioural test to examine whether hippocampal changes in BDNF and MR mRNA are associated with changes in spatial memory and learning, and whether developmental exposure to B (or other stressful protocols that mimic environmental stressful conditions) can contribute to alter these potential links upon adulthood.

The analysis of the gene signature of early life stress also suggested that hippocampal oxidative balance was altered in the adult B-treated phenotypes due to the down-regulation of both superoxide dismutase 3 (SOD3) and tyrosinase-related protein 1 (TYRP1) (Section 4.4.8.1, Figure 4.17). The sequences of these genes show a high degree of conservation across vertebrate groups and they are both related to the oxidative defence signalling pathway. In fact, SOD genes represent the first line of defence against the damaging effects induced by reactive oxygen species, converting superoxide radicals to hydrogen peroxide and water. TYRP1, in addition to its role in melanin synthesis (together with TYR and TYRP2) has a catalytic activity and participates at the secondary line of defence, which eventually detoxifies hydrogen peroxide into water and

oxygen (Scheiber, 2012). Therefore the decline of both SOD3 and TYRP1 suggest potential changes in antioxidant enzymatic levels possibly in response to elevated concentrations of reactive oxygen species in the hippocampus of the pre- and post-hatching B exposed birds. Given that the SOD genes identified so far (SOD1, SOD2 and SOD3) are highly compartmentalised in the cell (Parge *et al.*, 1992), the specific under-regulation of SOD3 mRNA is indicative of localised cellular oxidative responses within the extracellular space. Taken together, these data confirm the relevant associations between glucococorticoid hormones and oxidative stress (Costantini *et al.*, 2011a), but also reinforce the idea that overexposure to stress hormones can have the power to permanently influence the oxidative signalling cascade (Haussmann *et al.*, 2012; Marasco *et al.*, 2013 or Chapter 5 in this thesis). Given that oxidative stress is known to be implicated in a series of adult neurological diseases and can accelerate ageing processes, the long-term term implications of such alterations in the brain can possibly impinge on long-term survival and warrant further detailed investigations.

In contrast to the hippocampus, in the hypothalamus only a limited number of genes appeared to be responsive to pre –and post-hatching B in this study species (Section 4.4.8.2, Figure 4.20). The good clustering observed among these few genes, however, was promising and overall the enriched functional categories pointed to increased susceptibility to diseases, such as cancer. Although these results support the "Developmental origins of health and disease" phenomenon (e.g. Gluckman *et al.*, 2007), it is important to remark that the functional analysis used here is strongly biased by the biomedical literature, which rarely considers any possible beneficial and adaptive significance of the changes triggered in response to the priming effects of early life experiences.

4.5.1.2 Specific effects of pre- or post-hatching B

The experimental design and statistical approach used in this study allowed me to identify the gene expression patterns that were altered specifically by pre- or post-hatching glucocorticoid exposure. A global overview of the results clearly highlighted opposite magnitudes of the effects induced by the developmental B

protocols within the two tissues, with pre-hatching B showing a major impact in the hippocampal transcriptome and post-hatching B showing much larger effects in the hypothalamic transcriptome. Furthermore, the global gene expression directional changes associated with pre- or post-hatching B were opposite to one another. In fact, the genes' dynamic responses to pre-hatching B were skewed towards an overall repression of expression signals, while the responses to post-hatching B were markedly biased towards an up-regulation of the expression patterns.

Interestingly, the top significant biological function specifically altered by the elevation of yolk B in the hippocampus of the adult quail was involved in the development of the endocrine system (Section 4.4.8.1, Figure 4.18), with several key genes encoding hormone and neuropeptides receptors, such as the gene encoding aromatase (CYP19A1); melanocortin receptor 4 and 5 (MC5R and MC4R), both believed to have a role in energy homeostasis in the brain (Tao, 2010); transthyretin (TTR) and proenkephalin (PENK), which are both affected by glucocorticoids and are involved in developmental programming of the HPA axis in mammals (Fraser *et al.*,1997; Kohda *et al.,* 2006). Of particular interest was the down-regulation of the CYP19A1 in the pre-hatching B exposed birds (i.e. BC and BB groups) compared to the pre-hatching controls (i.e. CC and CB groups). This gene is a member of the cytochrome P450 super family and in a variety of vertebrate species it has been shown to have a pivotal role in phenotypic plasticity, neuroendocrine regulation and in the mediation of several behaviours, such as aggressive, emotional responses and song (non-mammalian models: reviewed by Forlano *et al.,* 2006; mammals: reviewed by Malone, 2013).The hippocampal expression signals of CYP19A1 transcripts in this study, however, were relatively low and the variation was high (5 < counts < 280). Given that RNA-seq is considered among the most sensitive technology for gene expression analysis, the reliability of these results should be further assessed using other techniques such as *in situ* hybridisation. Moreover, a note should be taken for TTR, the carrier of thyroid hormones and retinal binding protein in the cerebrospinal fluid. In fact, maternal separation stress has been shown to drastically decrease expression of TTR in the hippocampus of adult rat offspring (Kohda *et al.,* 2006; Wei *et al.,* 2012). More intriguingly, a recent microarray study in chicken suggested that this gene may be involved in the

transgenerational transmission of the brain transcriptome signature induced by unpredictable access to food (Nätt *et al.*, 2009). Given that the expression of TTR in the brain is controlled by MR and GR receptors (Martinho *et al.*, 2012), it is plausible that the hippocampal down-regulation of TTR in the pre-hatching B-treated birds might have been directly mediated by the gene MR, which as discussed above, was indeed affected across all the B-exposed phenotypes.

The functional analysis suggested a central role of the serotonin receptors (i.e. HTR2C, HTR3A and HTR1D) in the significant up-regulation of the genes that specifically responded to post-hatching B within the hypothalamus of the adult quail (Section 4.4.8.2, Figure 4.22). The crosstalk between serotonin neurotransmission and HPA axis, including the interactions of these two systems in the long-term effects of early life adversities and later susceptibility to neuroendocrine dysfunctions, has long been hypothesised (de Kloet *et al.*, 2005b). The results of this study provide experimental data supporting this hypothesis. Increases in serotonin have been reported in rats across various brain areas in response to different chronic stressors, such as foot shock or restraint (Inoue *et al.*, 1994, Adell *et al.*, 2006; respectively); adrenalectomy and exogenous supplementation of B have corroborated the primary role of glucocorticoids in these changes (Singh *et al.*, 1990). Although the neural circuitry mediating the links between serotonin and the HPA axis systems remain to be fully clarified, a recent study in mice suggested important regulatory interactions between serotonin and corticotrophin-releasing hormone signalling systems via the activation of HTR2C within the hypothalamus (Heisler *et al.*, 2007). The latter findings support the results from the cluster analysis reported in this study that linked the serotonin receptor pathway with the up-regulation of one type of corticotrophin releasing hormone receptor 2 (CRHR2). Taken together, these data suggest that post-hatching exposure to B during development shaped, in the long-term, the brain serotonergic system, possibly via transcriptional changes mediated by corticotrophin-releasing hormone. This idea is supported at least in part by a recent study in the chicken showing that exposure to high dose of pre-hatching B via yolk injections caused long-lasting modifications in both the hypothalamic serotonergic genes (including up-regulation of HTR1A and down-regulation of the serotonin biosynthetic enzyme tryptophan hydroxylase) and HPA axis genes (including down-regulation of

corticotrophin-releasing hormone); interestingly, these gene expression changes were correlated with increases in aggressive behaviour, providing evidence of reciprocal links between the neurocircuits for stress physiology and aggression (Ahmed *et al.*, *in press*). In future studies it will be extremely important to couple investigations at the gene expression level with behavioural observations to further our understanding of the functional links between genes, hormones and behaviour.

4.5.1.3 Interactive effects of pre- and post-hatching B

Surprisingly, a small number of interactive genes were altered by the combined exposure to pre- and post-hatching exogenous B in both the hippocampus and hypothalamus. However, the results in the hippocampus seemed interesting. In fact, the overall down-regulation in gene expression patterns observed in both the pre-hatching (BC) or post-hatching B-exposed birds (CB) seemed augmented in the BB birds and appeared to have altered both GABA and glutamate neuronal receptor signalling (Section 4.4.8.1) and molecular transport pathways via solute carrier transporters (Figure 4.19). These data suggest that cumulative long-lasting modifications in synaptic communication may have occurred in response to the combined stressful treatments and the biological relevance of such changes should be further investigated in future studies. Another significant result relates to the opposite effect of pre- and post-hatching B in vasotocin-neurophyin VT (AVT) (Section 4.4.7.1, Figure 4.13-VI), with pre-hatching B decreasing expression and post-hatching B potentiating expression. These opposite gene responses were cancelled out in the BB birds, which showed AVT mRNA levels similar to the adult controls. Abundant evidence has shown that AVT is a key gene in the regulation of the HPA axis activity and behaviour across vertebrate species (reviewed by Goodson and Bass, 2000). A recent study in mice has demonstrated that the regulation of AVT is permanently altered by early life stressful events via DNA methylation changes at specific key promoter regions of this gene in the paraventricular nucleus of the hypothalamus (Murgatroyd *et al.*, 2009). In the present experiment, however, AVT gene expression in the hypothalamus was not affected by the B-treatments. I also point out that while AVT was hugely expressed within the hypothalamus across all the treatment groups (19179.1 ± 2158.5 counts), much lower gene expression levels were

detected within the hippocampus (566.1 ± 303.8 counts). Such tissue-specific gene expression differences were expected as AVT is known to be predominantly produced within the hypothalamus (see Chapter 1, Paragraph 1.2 and Figure 1.2). Despite line of evidence indicating the presence of extra-hypothalamic AVT fibers within prosencephalic and mesencephalic areas in the chicken and Japanese quail brain (e.g. Panzica *et al.*, 1986; Panzica *et al.*, 1988), I am unable to define to which extent these low hippocampal AVT expression levels detected in this study will correspond to an actual production of AVT. Nevertheless, the simultaneous down-regulation of hippocampal AVTR2 observed in both the pre- and post-hatching B-treated birds (Paragraph 4.4.8.1) suggests potentially important functional changes in the vasotonergic transcriptional regulatory mechanisms as a consequence of the developmental B exposure that should be further validated in future studies. As discussed in detail in the Paragraph 5.5.2 below, it will be important in future studies to use a more specific brain dissection method in individual-non pooled samples to reduce biological variability, increase statistical power and investigate potential sex-specific treatment effects.

4.5.2. Further technical considerations and limitations of the study

There are a number of technical limitations that must be noted when considering the results from this study.

First, the alignment of the quail RNA-seq reads was performed on the chicken genome. Although the use of the chicken genome as a reference was the best choice here, the inter-specific alignment approach imposed a shrinking of the initial read length of 50% (Figure 4.5). Furthermore, approximately 40% of the reads were lost post-alignment as a consequence of annotation differences between the quail and the chicken genome (Table 4.2). Such reduction of the initial available information in the raw data might have significantly reduced the depth of coverage of the quail reads to the reference. Reduced depth of coverage has been shown to decrease sensitivity and accuracy of the differential

gene expression analyses (Tarazona *et al.*, 2012). The *de novo* assembly of the quail reads would overcome this constraint as this approach does not necessarily need the use of a reference genome and would also be particularly useful to identify novel quail-specific transcripts. However, the assembly *de novo* is complex and computationally intensive. For this reason, this was not a viable option for this project. Importantly, the packages available to date are not limitation-free, especially with Illumina generated data (as in this study) due to (1) the higher base-calling error rates, and (2) the short-length of the reads relative to other sequencing platforms (e.g. 454), which can constraint the correct concatenation of the contigs (Francis *et al.*, 2013).

Another important limitation for the differential statistical analyses was the high intra-replicate variation observed across the treatment groups within a specific tissue (Figure 4.7). There may be several reasons to explain such high variation. For instance, the analysis was conducted on pooled samples containing both males and females. The inclusion of both the sexes within each biological pool constrained my ability to investigate sex-specific gene expression differences (and potential interactions with treatment) and might have increased biological range of variation. Furthermore, in this study I used the whole hippocampus and hypothalamus. In birds the region defined as the hippocampus is a V-shaped structure composed of a nearly homogeneous arrangement of densely packed neurons which progressively merge into the parahippocampal region without precise boundaries (Karten and Hodos, 1967; Krebs *et al.*, 1989; Gupta *et al.*, 2012). Although extreme care was taken during the dissections, which were always performed with the use of the brain topography of the chicken brain atlas, it is likely that the hippocampal punches contained also some parahippocampal neurons adjacent to the hippocampus. Moreover, the hypothalamus is known to encompass several specific nuclei. Discrete and specific transcriptome signatures across differing nuclei within the same neural structure have been reported in rats (Gautvik *et al.*, 1996). As a consequence, the high variation in the RNA samples observed in this study might be associated with potentially different gene expression patterns between the two main sub-divisions in the hippocampal complex (i.e. hippocampus and parahippocampus) and across the different hypothalamic nuclei. Furthermore, the PCA graphs suggested lower within replicate variation of the gene expression signals in the

birds that were specifically treated *in ovo* (i.e. BC) compared to those that experienced post-natal developmental stress (i.e. CB and BB). The post-hatching doses of B employed here were carefully validated in pilot work. In this experiment the juvenile quail received a fixed daily amount of exogenous B according to the specific post-hatching age range. However, it is possible that individual variation in body mass across the birds may have produced different effects on the HPA axis sensitivity. In contrast, body mass trajectories of the embryos are likely to be much less variable compared to those in the hatchlings due to the space constraint imposed by the egg. Future studies, therefore, may wish to consider the use of a better standardised oral hormonal administration protocol by adjusting the doses by daily body mass values of each individual bird. However, a mass-scaled approach would also mean frequent handling, and the consequent habituation and dampening of the HPA axis that this is likely to generate should be carefully considered when designing the experiment. Finally, as the magnitude of the B treatment on the brain transcriptome signature was not high it would be sensible in future studies to conduct global gene expression analysis at the individual level. This approach was not a viable option for the present work due to the high costs of the analyses, but the progressive reduction in the sequencing costs may make this option practically possible in future studies.

A third aspect that needs further discussion regards the statistical analysis. Differential gene expression analysis of RNA-seq generated data are a well known challenging task in bioinformatics and the available statistical packages are still under development. The results from the pair-wise statistical comparison across Cufflinks, Bayseq and RankProducts showed an overall good reproducibility between Cufflinks and RankProducts, but not between these two and Bayseq (Figures 4.8-4.11). In fact, Bayseq hardly detected differentially expressed genes except for a few treatment contrasts in which between-replicate variation was relatively small (i.e. BC *vs* CC in the hippocampus and BB *vs* CC in the hypothalamus). Bayseq was even more conservative in the models that considered the four treatments simultaneously, possibly because of the increased variance in the data. Bayseq's poor performances may, therefore, be linked to the large variation across biological replicates, which may have limited the derivatisation of the empirically determined prior distribution from the

dataset. This statistical package has been shown to give meaningful better performances than other existing methods (including Cufflinks) when the datasets have (1) approximately constant dispersion, (2) a large proportion of differential expressed genes, or (3) unidirectional differential expression, with all the differentially expressed genes down- or up-regulated (Hardcastle and Kelly, 2010; Kvam *et al.*, 2012). None of these three criteria appear to be satisfied in my data. Regardless, the latter studies did not take into account the variability of biological replication, which is frequently high in RNA-seq data due to the low (if any) number of replicates and has a significant impact on gene calling performances (Zheng, 2012). For instance, recent studies propose that non-parametric statistics may control better for false positive rates than parametric methods in datasets with large biological variation (Zheng, 2012; Tarazona *et al.*, 2012). This would then suggest that RankProducts statistics may be a more flexible and more data-adaptive tool than the other inferential methods, such as Cuffdiff and Bayseq for RNA-seq differential expression analysis. Furthermore, RankProducts tended to be less conservative than Cuffdiff and found larger number of differentially expressed candidates in most of the contrasts. These data appear to suggest that RankProducts may be a powerful statistical method also with RNA-seq data, as previously demonstrated with microarray-generated data (Breitling *et al.*, 2004; Breitling and Herzyk, 2005; Jeffery *et al.*, 2006). More work with real and simulated data, taking into account the coefficient of variation across biological replicates, would be extremely useful to further our understanding of the applicability of RankProducts in RNA-seq. As I could not exclude the possibility that RankProducts analysis may also have less control of type 1 error and increase in false positives rates, I further filtered the data using the Vector Analysis in accordance with the relevant biological questions of the study. Importantly, this tool allowed me to (1) control for the biological variability by identifying the consistent genes' dynamic responses to the early life treatments across the pair-wise contrasts, and importantly to (2) overcome the limitation due to the comparisons between groups in a one-way layout.

The validation analysis was conducted using both microarrays and qPCR on specific candidate genes. The correlation of the absolute expression signals between Microarray and RNA-seq was significant, but the coefficient of

correlation was not very high (approximately 0.60). However, these results are in line with other published work (e.g. Mortazavi *et al.*, 2008; Chen *et al.*, 2011; Brennan *et al.*, 2012). In fact, consistent with the latter previous work, the correlation was not linear and there was a slight compression in the microarrays data at the high end. The scatter increased at the low expression values, which, again, was not surprising as background correction methods for microarrays are known to be complicated when signal levels approach the noise levels (Ramdas *et al.*, 2004). Another aspect to point out is that there was an overall poor agreement between the two platforms at the level of the differential statistical analysis. RNA-seq tended to detect larger number of differentially expressed genes, which was expected as it is known to be more sensitive than microarrays (Mortazavi *et al.*, 2008). Other than the lower general sensitivity of microarrays, however, it must be noted that the chicken annotation release used for RNA-seq data was more recent than the annotation release used for the microarray data. This was something I could not control for because the library files are provided by Affymetrix and they were downloaded just before starting the analysis. In this study, I merged the datasets obtained by the two technologies using the Ensembl Identifiers. However, while all the genes from RNA-seq data had an Ensembl Identifier, many probe-sets did not and were provided with only the official gene names. All these genes were excluded from the comparison. Therefore it is likely that these annotation differences underestimated the real actual agreement between the two platforms.

Finally, as expected, the qPCR data on the 5 candidate genes showed highly similar expression signals and fold changes across the treatment groups with both RNA-seq and Microarrays.

4.6 Conclusion

The genome-wide results obtained in this study suggest that early life stressful condition mimicked via physiological overexposure to B have the potential to induce distinct brain tissue-specific modifications in adult transcriptome signature in the hippocampus and hypothalamus of the Japanese quail. This

study reinforces the importance of well known key genes for the control of the HPA axis and brain development. Importantly this is the first experimental attempt to disentangle the specific or combined long-lasting effects of pre- and post-hatching exposure to B on gene expression patterns. The data in this respect contribute to novel knowledge on the overall transcriptional regulation and functional trends of developmental glucocorticoid programming, emphasising the importance of considering the effects of interactive environmental cues across differing developmental periods as these may induce both cumulative and opposing gene expression responses in the brain. Future studies will be needed to test if these changes are associated with changes in reproductive performances and life expectancy in order to further our understanding of the potential adaptive or maladaptive significance of developmental stress programming.

5. Chapter

Developmental post-hatching stress can alter the effects of pre-hatching stress on the adult redox balance

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5.1 Abstract

Across diverse vertebrate taxa, stressful environmental conditions during development can shape phenotypic trajectories of developing individuals, which, while adaptive in the short-term, may impair health and survival in adulthood. Regardless, the long-lasting benefits or costs of early life stress are likely to depend on the conditions experienced across differing stages of development. Here, I use the Japanese quail to experimentally manipulate exposure to the glucocorticoid hormone corticosterone (B) in developing individuals. I test the hypothesis that interactions occurring between pre- and post-hatching developmental periods can induce long-term shifts in the adult oxidant phenotype in non-breeding sexually mature individuals. I show that developmental exposure to B can induce long-term alterations in the basal antioxidant defences. The magnitude of these effects depends upon the timing of glucocorticoid exposure and upon interactions between the pre- and post-hatching B. I also find differences among tissues with stronger effects in the erythrocytes than in the brain in which the long-term effects of glucocorticoids on antioxidant biomarkers appear to be region-specific. Recent experimental work has demonstrated that developmental exposure to stress hormones can markedly reduce adult survival. The results from this study suggest that long-

term shifts in basal antioxidant defences might be one of the potential mechanisms driving such accelerated ageing processes and that post-natal/hatching interventions during development may be a potential tool to shape the effects induced by pre-natal/hatching glucococorticoid-exposed phenotypes.

5.2 Introduction

Early life events can drive phenotypic traits of developing individuals (Mousseau and Fox, 1998; Monaghan, 2008). A growing scientific interest focuses on furthering our understanding of the long-term effects associated with poor quality developmental environments on important phenotypic traits that can impact health and adult survival. Pioneering studies in mammals have linked a variety of perinatal stressors (e.g. intrauterine growth restriction, maternal separation, reduced maternal care and child abuse) with persistent metabolic changes in the developing individuals that are thought to be important in determining adult health outcomes (for recent reviews see Meaney *et al.,* 2007; Cottrell and Seckl, 2009). Changes in adult phenotypes in response to stressful developmental conditions have now been reported in a broader range of vertebrate taxa (e.g. fish: Roche *et al.*, 2012; reptiles: De Fraipont *et al.,* 2000; birds: Monaghan *et al.,* 2012). It is now widely believed that "developmental programming" may reflect a conserved biological phenomenon across vertebrate species, with significant consequences for a range of health indicators in later life (Love *et al.*, 2013).

Glucocorticoid stress hormones are the main candidates as mediators of developmental stress programming (Seckl, 2004). Growing individuals are exposed to glucocorticoids during their pre-natal/pre-hatching development, primarily via maternal routes (e.g. McCormick, 1999; Hayward and Wingfield, 2004; see also review by Henriksen *et al.*, 2011) or during post-natal/post-hatching development, for instance via the direct effects of environmental stressors on their own physiological systems (e.g. Meylan *et al.,* 2002; Spencer *et al.,* 2009). It has been proposed that maternal stress hormones induce

anticipatory responses in the embryo that could prime its phenotype to better cope with future post-natal/hatching environmental stimuli (Bateson *et al.*, 2004; Gluckman and Hanson, 2004). However, delayed costs may arise because of inevitable physiological constraints (i.e. "*silver spoon hypothesis*", Grafen, 1988), for example those associated with poor maternal conditions, or because of a mismatch between the predicted and the encountered post-natal/hatching environmental conditions (i.e. "*the mismatching hypotheses*", reviewed by Monaghan, 2008; see also Hales and Barker, 2001). Acute and persistent exposure to stress hormones can be damaging for key self-maintenance processes, such as energetic metabolism, cellular differentiation, myelination, apoptosis or neurogenesis (Sapolsky *et al.,* 1990; de Kloet *et al.,* 2005a). Oxidative stress, a condition of unbalance between the products of oxygen metabolism (i.e. reactive oxygen species) and the individual's capacity to contrast/ease their damaging effects, may play a key role in mediating these long-term costs. In fact, oxidative stress can lead to the production of biomolecular oxidative damage to cells (Halliwell and Gutteridge, 2007; Costantini and Verhulst, 2009) and is implicated in cell senescence and neurodegenerative disorders (Finkel and Holbrook, 2000). Prolonged exposure to exogenous glucocorticoids promotes cellular oxidative stress in the body (e.g. McIntosh and Sapolsky, 1996; McIntosh *et al.,* 1998; Costantini *et al.,* 2011a). A recent meta-analysis showed that the magnitude of these effects significantly differs among tissues, with the brain and blood showing respectively high and moderately high effect sizes (Costantini *et al.*, 2011a). Further, these effects change across an individuals' life cycle, with juvenile stressed individuals being more vulnerable than adults (Costantini *et al.*, 2011a).

The hypothesised links between early life stress and shifts in an individual's basal oxidative balance is beginning to be explored (Haussmann and Marchetto, 2010; Haussmann *et al.,* 2012). For example, in marmoset monkeys (*Callithrix jacchus*) maternal overexposure to dexamethasone led to enhanced gene expression of antioxidant defences in the aorta of adult offspring and these effects were more pronounced when the hormone was administered during the later stages of gestation compared to the earlier stages of gestation (Atanasova *et al.*, 2009). In the chicken (*Gallus gallus*) *in ovo* exposure to corticosterone (B, the primary glucocorticoid in birds) produced significant increases in

oxidative damage and cell senescence rate at three weeks post-hatching compared to control birds (Haussmann *et al.*, 2012). However, the "molecular imprinting" initiated in the stressed embryos is likely to be plastic and mediated by the environment encountered at birth/hatching and throughout post-natal/hatching development (Monaghan, 2008). Therefore, the degree of developmental plasticity and the long-term consequences arising from these potential adjustments may depend on the nature of both pre- and early post-natal/hatching cues. For instance, pre- and post-natal/hatching stressful developmental conditions may have cumulative effects on cellular energetic state, exacerbating oxidative insults to tissues as a result of additive physiological constraints or stimulating investment in antioxidants to prevent damage to biomolecules. The potential long-lasting effects of such interactions have hitherto been untested.

The aim of the present study was to examine the long-term potential interactive effects of pre- and post-hatching physiological exposure to elevated B on adult oxidative status in the Japanese quail. More specifically, the main objectives were to analyse whether early life stress would induce long-lasting alterations to adult body oxidative defences, ultimately causing oxidative damage. These objectives were accomplished by measuring enzymatic (i.e. superoxide dismutase, glutathione peroxidase) and non-enzymatic (i.e. total non-enzymatic antioxidant capacity) antioxidant biomarkers, as well as protein carbonyl content as a marker of oxidative damage, in both the blood and brain tissues in adult non-breeding quail between 9-10 weeks of age (puberty in this species is reached between 6-8 weeks of age, Ottinger, 2001). Measurements in the blood and the brain allowed me to estimate body oxidative status in two target tissues of body oxidative balance in both proliferating and non-proliferating (i.e. post-mitotic) cells, respectively. The highly precocial nature of the Japanese quail, allowed me to independently manipulate B concentrations in the egg yolk and/or in the endogenous circulation of the hatchlings during the linear phase of growth. This is the first experiment that was specifically designed to study the effects of pre-hatching conditions under differing post-hatching developmental environments in the absence of the potential confounding factors of maternal care and, hence, appropriately testing the hypothesis of key interactions affecting phenotypic plasticity during developmental periods. I predicted that

the developmental B manipulations would lead to changes in body oxidative balance via modifications in the antioxidant lines of defences (Atanasova *et al.*, 2009), which in turn may be linked with increased biomolecular damage (Haussmann *et al.*, 2012). Furthermore, in light of the high vulnerability of the nervous system to oxidative stress (e.g. Halliwell, 1992) and the results observed in the recent meta-analysis mentioned above (Costantini *et al.*, 2011a), I expected that the early life treatments would induce a stronger effect in the brain than in the blood.

5.3 Materials and methods

5.3.1 Pre- and post-hatching hormonal manipulation

The birds used in this study are part of the main experiment described in detail in Chapter 3 (Section 3.3.1). Briefly, pre-hatching stress exposure was mimicked by injecting a physiological dose of B into the yolk of fertile eggs at day 5 of incubation, whilst post-hatching (PN) stress was mimicked via daily oral administration of a physiological dose of B to the quail hatchlings from PN5 to PN19. The experiment was repeated twice (Batch 1 and Batch 2).

5.3.2 Measuring the long-term effects of early life hormonal manipulation on oxidative status

5.3.2.1 Tissue collection and brain dissections

At day PN64, blood samples (taken within 1.5 min of opening the cage) were collected as described in Chapter 3 (Section 3.3.2.2) and, here, the red blood cells were used to measure oxidative stress biomarkers (see Section 5.3.2.2 below). Erythrocytes are considered to be a valid group of cells for the measurement of oxidative stress due to their high content of oxygen and haemoglobin (Pandey *et al.*, 2011). Furthermore, recent experimental work demonstrated a strong correlation between antioxidant biomarkers measured in

red blood cells and plasma, with a parallel co-variation between these two blood compartments both in the enzymatic (i.e. glutathione peroxidase) and non-enzymatic antioxidant capacity at the individual level (Costantini *et al.,* 2011b). Therefore the measurements in the red blood cells can reliably give an overall indication of the cellular redox status in the blood circulation.

Between days PN69-73, the birds were sacrificed and the brains dissected as described in detail in Chapter 4 (Sections 4.3.1, 4.3.2). For the present study, two equivalent bilateral punches (2 mm diameter each) surrounding the lateral ventricle and including both telencephalic (i.e. nidopallium) and diencephalic thalamic nuclei (i.e. dorsolateral anterior nuclei) (herein referred as midbrain) were obtained (Figure A1, Appendix). Subsequently, the cerebellum was also dissected out. Tissues from midbrain and cerebellum were stored separately in collection tubes and placed back to -80 ºC until analyses. The midbrain samples were chosen because the main purpose of the study was to obtain a general measurement of oxidative status in the brain rather than in one specific brain nucleus; the punch technique allowed me to precisely standardise the position of the punches across the experimental birds. As the effects of glucocorticoids on cellular oxidative state can spatially vary in the brain (McIntosh *et al.,* 1998), by taking also the cerebellum samples I ensured a replicated measurement from each individual bird. Brain tissues from 2 females (1 in the BC group and 1 in the CB group) could not be dissected out and, therefore, were excluded from the later analyses; red blood cells from 1 female (BC group) were missing and this individual was also excluded from the analyses.

5.3.2.2 Laboratory analyses

In each tissue that was collected 4 oxidative biomarkers were measured: superoxide dismutase (SOD); glutathione peroxidase (GPX), non-enzymatic antioxidant capacity (OXY), and protein carbonyls (PC). SOD, GPX and OXY are established indicators of antioxidant defences preventing oxidation of cell components; while PC measures the degree of protein carbonylation and is considered a reliable proxy of cellular oxidative protein damage. Japanese quail

reach puberty between 6-8 weeks of age (Ottinger, 2001). Quail used in this study were sampled between 9-10 weeks of age and were fully grown and capable of breeding if they would have been stimulated with an appropriate reproductive induction protocol (Robinson and Follett, 1982). Therefore, my sampling schedule was a reliable long-term measurement of the effects of early life stress on individuals' redox physiology in non-breeding sexually mature individuals.

Midbrain and cerebellum tissues were homogenised in ice cold PBS (pH = 7.19-7.59; molarity = 0.150M; supplemented with 20% (v/v) of glycerol and with 0.2mM of phenylmethylsulfonyl fluoride as an inhibitor of proteases) using a pestle and mortar. Samples were then sonicated for 10min and then centrifuged for 10min at 10,000rpm. The supernatant was split into different aliquots, which were stored at -80°C for later analyses. Haemolysates were centrifuged to separate cell membranes from the supernatant, which were used immediately for the analyses. Preliminary tests were conducted to determine the appropriate dilution factors in order to assure that each biomarker across the different tissues was within the linear range of the assay. A Thermo Scientific Multiskan Spectrum (ThermoFisher, Vantaa, Finland) was used to read the absorbance of the assay reactions.

The Ransod assay (RANDOX Laboratories, Crumlin, UK) was used to quantify the concentration of SOD. As shown in Figure 5.1, this enzyme is involved in the first step of the antioxidant enzymatic cascade catalysing the dismutation of superoxide radical into oxygen and hydrogen peroxide. The assay employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. SOD is then measured by the degree of inhibition of this reaction. Red blood cells and homogenates of midbrain and cerebellum were diluted 1:600, 1:50 and 1:100 with distilled water, respectively. The assay laboratory steps were performed following the Manufacturer's instructions. The assay was adapted to 96-well plate readers (Corning Life Sciences, Amsterdam, NL) by scaling down the volume of the assay reagents and experimental samples by a factor of 2.5. This allowed a reduction in the volume of tissue samples to 6µl. Values were calculated using a calibration curve for each assay. Analyses were

run in duplicate and the mean coefficients of intra- and inter-assay variation were 5.8 and 6.9%, respectively.

The Ransel assay (RANDOX Laboratories, Crumlin, UK) was used to quantify the concentration of GPX. This peroxidase decomposes hydrogen peroxides resulting from SOD activity and other cellular processes in water and molecular oxygen by oxidising the reduced form of glutathione (Figure 5.1). The assay laboratory steps are based on the original method (see Paglia and Valentine, 1967) and analyses were carried out according to previous studies (e.g. Costantini *et al.*, 2011b). The samples were diluted 1:40 using the diluting agent provided by the Manufacturer. The assay was adapted to 96-well plate readers (Corning Life Sciences, Amsterdam, NL) by scaling down the volume of the assay reagents and experimental samples by a factor of 5 (the volume of tissue sample used in the assay was 4µl). Analyses were run in duplicate and the mean coefficients of intra- and inter-assay variation were 6.5 and 7.3%, respectively.

The OXY-Adsorbent test (Diacron International, Grosseto, Italy) was used to quantify the capacity of non-enzymatic antioxidant compounds (OXY) present in the sample to cope with the *in vitro* oxidant action of hypochlorous acid (HOCl; an endogenously-produced oxidant). The OXY assay measures a variety of non-enzymatic antioxidants, including vitamins, carotenoids, flavonoids and, most importantly glutathione, which is present in millimolar concentrations in animal cells; in fact OXY significantly correlates with thiols in the blood ($r = 0.65$-0.67, Palleschi *et al.*, 2007). Importantly, a recent longitudinal study in a wild bird population has suggested that the OXY assay is a biological predictor of long-term survival (Saino *et al.*, 2011). Red blood cells and homogenates of midbrain and cerebellum were diluted 1:600, 1:50 and 1:35 with distilled water, respectively. The procedure was carried out following the Manufacturer's instructions (see also Costantini *et al.*, 2011b). The assay was adapted to 96-well plate readers (Corning Life Sciences, Amsterdam, NL) by scaling down the volume of the assay reagents and experimental samples by a factor of 5 (the volume of tissue sample used in the assay was 200µl). The absorbance was read at a wavelength of 490 nm. Values were calculated according to a reference standard. Analyses were run in duplicate and the mean coefficients of intra- and inter-assay variation were 5.4 and 7.3%, respectively.

Protein carbonyls (PC) were measured according to Levine (Levine et al., 1990; see also Cao and Cutler, 1995; Montgomery et al., 2011). Carbonyl groups are introduced into the proteins from free radicals or via reactions with lipid peroxidation products or carbohydrates (Figure 5.1); protein carbonylation is mostly irreversible (Halliwell and Gutteridge, 2007). Nucleic acids were removed by adding 1 volume of a 10% solution of streptomycin sulfonate to 9 volumes of sample. PC were derivatised to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH). The pellet was precipitated with cold trichloroacetic acid at 20% and then washed three times with a solution 1:1 of cold ethanol-ethyl acetate. The pellet was finally re-suspended in 350µl of 6M guanidine hydrochloride. The absorbance was read at 370nm. Analyses were run in duplicate and the mean coefficients of intra- and inter-assay variation were 9.0 and 12.3% respectively.

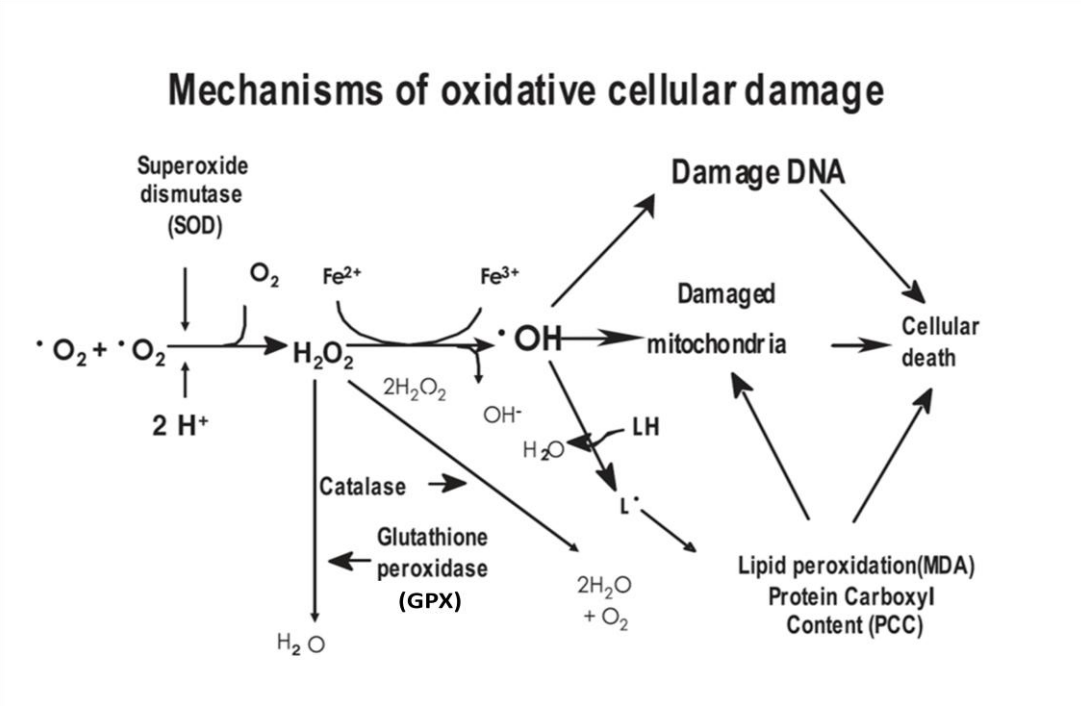

Figure 5.1 Mechanisms of oxidative cellular damage (from Morón and Castilla-Cortázar, 2012). Free radicals are reduced into water via the action of the enzyme superoxide dismutase (SOD) first, and then of Catalase and gluthathione peroxidise (GPX). The generation of hydroxyl radicals from hydroperoxide leads to the development of oxidative cell injury: DNA damage; carboxylation of proteins; and lipid peroxidation, including the lipids forming mitochondrial membranes. By these pathways, oxidative damage can eventually lead to cellular death.

Measures of all the biomarkers were then standardized by expressing the concentrations per mg of proteins as measured by the Bradford protein assay (Bio-Rad Laboratories, Hercules, USA) using a standard curve of bovine serum albumin. Red blood cells and the homogenates of midbrain and cerebellum were diluted 1:600, 1:16 and 1:16, respectively with distilled water. For PCs, all samples were first diluted with distilled water in order to have a concentration of 1mg of protein per ml. Analyses were run in duplicate and the mean coefficients of intra- and inter-assay variation were 4.8 and 5.8%, respectively.

5.3.3 Statistical analysis

Analyses were performed in PASW statistics, 18.0.0 (SPSS, Inc., 2009, Chicago, IL) using Linear Mixed Effect models (LMEs) fitted by Restricted Maximum Likelihood. Data were analysed by tissue and separately for each biomarker. In all LMEs, the fixed factors were pre-hatching treatment, post-hatching treatment, sex, and all the two- and three-way interactions; while batch and maternal identity were entered as random factors to control for sources of variation between the two batches and pseudo-replication, respectively. Non-significant effects ($p > 0.05$) were removed from the models following a backward procedure (Crawley, 1993). To meet the assumptions of the LME, SOD in the red blood cells and PC in the midbrains were square root-transformed for normality; cerebellum GPX was $\log_{10}$-transformed to improve normality. All model residuals were normally distributed. Unless otherwise specified, the data are presented as means ± s.e.m.

5.4 Results

5.4.1 Red blood cells

Descriptive statistics for each oxidative stress biomarker across the treatment groups and separately by sex is presented in Table 5.1a. There were no significant treatment or sex effects on SOD in the red blood cells (Table 5.2a for

full statistics). Developmental exposure to B had a significant effect on red blood cell GPX in the adult quail (pre-hatching treatment: $F_{1,67.58} = 4.87$, $p = 0.031$; post-hatching treatment: $F_{1,54.62} = 5.47$, $p = 0.023$, respectively) explained by up-regulated enzymatic activity in the early B-exposed birds compared with the controls (Figure 5.2a). There were no significant interacting effects between the pre- and post-hatching treatment, or among the B treatments and sex in this variable; there was no effect of sex as a main factor (Table 5.2a). I found a significant interaction between the pre- and post-hatching treatment explaining OXY in the red blood cells ($F_{1,65.50} = 5.75$, $p = 0.019$, Figure 5.2b). This was due to lower OXY in all the B-exposed birds compared to the controls, but overall this reduction was less pronounced in the BB birds compared to the BC or CB birds (Figure 5.2b). None of the other factors in the model were significant (Table 5.2a). PC were significantly higher in females compared to males ($F_{1,69.28} = 7.80$, $p = 0.008$; females: 12.24 ± 0.96 ; males: 8.48 ± 0.81), but the concentration of PC was unaffected by exposure to B, and none of the interactions among the B treatments and sex were statistically significant (Table 5.2a).

5.4.2 Brain

Descriptive statistics for each oxidative stress biomarker across the treatment groups and separately by sex is presented in Table 5.1b, c. There were no significant treatment or sex effects on any biomarker measured in the midbrain samples (Table 5.2b). In the cerebellum, GPX was marginally up-regulated in birds that experienced post-hatching exposure to B ($F_{1,\ 63.84} = 3.57$, $p = 0.063$), but not in the birds that were exposed to the pre-hatching B treatment alone ($F_{1,\ 70.43} = 1.11$, $p = 0.297$); none of the other factors were statistically significant (Figure 5.3a; Table 5.2c). I did find a significant interaction between the pre- and post-hatching B treatment explaining cerebellum OXY ($F_{1,66.20} = 4.428$, $p = 0.039$; $p > 0.1$ for all the other factors, see Table 5.2c) due to lower OXY in the BB birds in contrast with the pattern observed in the BC or CB birds (Figure 5.3b).

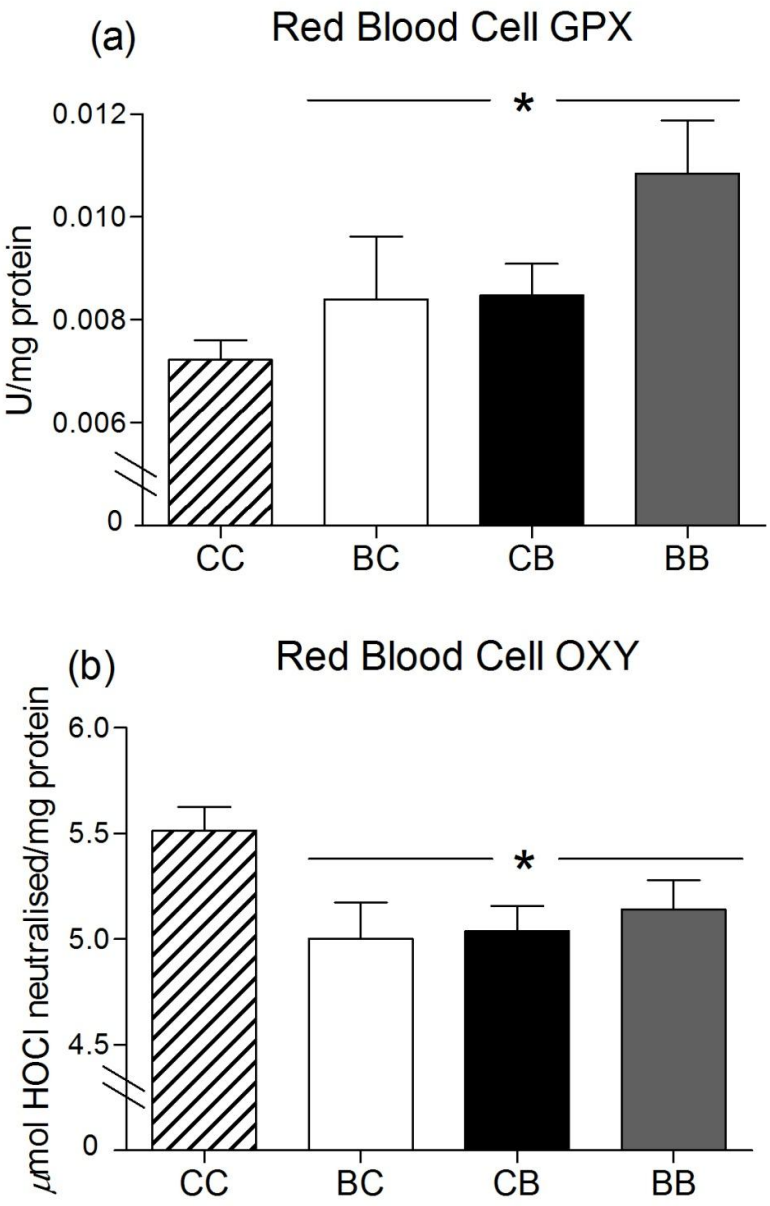

Figure 5.2 The effects of physiological overexposure to corticosterone (B) during the pre- and/or post-hatching development on (a) red blood cell glutathione peroxidase (GPX) and (b) red blood cell non-enzymatic antioxidant capacity (OXY) in adult Japanese quail. (a) The B-treated quail (BC, CB, BB groups) showed overall higher GPX activity than the CC group, and these effects were more pronounced in the BB quail (Linear Mixed Model: pre-hatching treatment, p = 0.03; post-hatching treatment, p = 0.02); whereas OXY levels were significantly lower in all the B-treated birds compared to the CC group, but this reduction was less pronounced in the BB birds (Linear Mixed Model: pre-hatching x post-hatching interaction, p = 0.02). On both graphs, * denotes $p < 0.05$. CC = pre-hatching untreated and post-hatching untreated birds; BC = pre-hatching B-treated and post-hatching untreated birds; CB = pre-hatching untreated and post-hatching B-treated birds; BB = pre-hatching B-treated and post-hatching B-treated birds. Sample sizes: CC: females = 9, males = 14; BC: females = 8, males = 6; CB: females = 9, males = 10; BB: females = 9, males = 9. Data represent un-transformed means ± s.e.m.

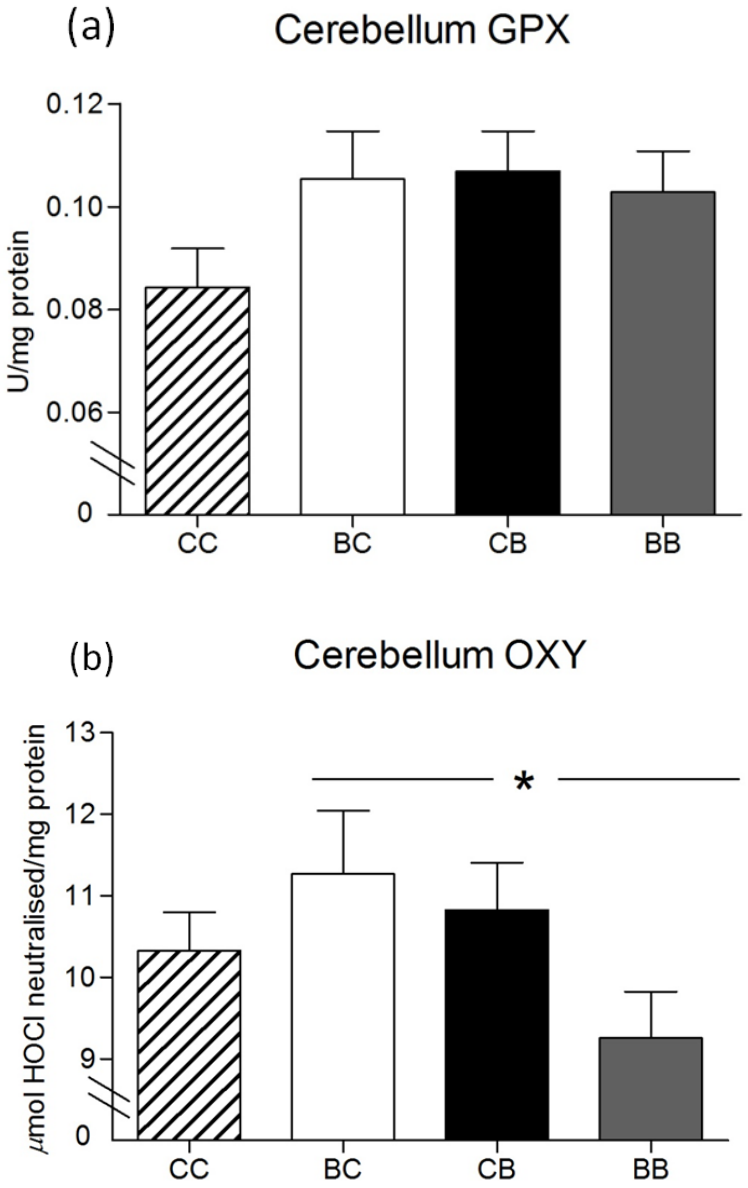

Figure 5.3 The effects of physiological overexposure to corticosterone (B) during the pre- and/or post-hatching development on (a) cerebellum glutathione peroxidase (GPX) and (b) cerebellum non-enzymatic antioxidant capacity (OXY) in adult Japanese quail. (a) Cerebellum GPX was marginally up-regulated in the birds that were treated with B post-hatching (CB and BB groups) compared to the CC birds (Linear Mixed Model: post-hatching treatment, $p = 0.06$); (b) Cerebellum OXY in the BB birds was significantly lower compared to the levels in the BC or CB birds (Linear Mixed Model: pre-hatching x post-hatching interaction, * $p = 0.04$). CC = pre-hatching untreated and post-hatching untreated birds, BC = pre-hatching B-treated and post-hatching untreated birds; CB = pre-hatching untreated and post-hatching B-treated birds; BB = pre-hatching B-treated and post-hatching B-treated birds. Sample sizes: CC: females = 9, males = 14; BC: females = 8, males = 6; CB: females = 9, males = 10; BB: females = 9, males = 9. Data represent un-transformed means ± s.e.m.

Table 5.1 Mean ± s.e.m. of the oxidative stress biomarkers (superoxide dismutase, SOD; glutathione peroxidase, GPX; non-enzymatic antioxidant capacity, OXY; and protein carbonyls, PC) across the different treatment groups (CC, BC, CB, BB) measured in (a) the red blood cells, (b) midbrain, and (c) cerebellum tissues in the adult Japanese quail (post-hatching day 64-73), separately by sex.

(a) Red Blood Cells

Female:	CC		BC		CB		BB	
Biomarker	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
SOD	0.8871	0.1088	0.7677	0.1376	0.8272	0.1433	0.7912	0.1182
GPX	0.0080	0.0004	0.0092	0.0018	0.0085	0.0008	0.0126	0.0016
OXY	5.5022	0.2301	5.1564	0.2444	5.1000	0.1979	5.3088	0.1754
PC	13.2553	2.2177	11.4723	1.8916	10.0878	1.4878	14.0637	2.0300

Male:	CC		BC		CB		BB	
Biomarker	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
SOD	0.6490	0.0675	0.6657	0.0477	0.7168	0.0798	0.7772	0.0861
GPX	0.0067	0.0006	0.0074	0.0016	0.0085	0.0010	0.0091	0.0010
OXY	5.5204	0.1201	4.7992	0.2231	4.9830	0.1495	4.9717	0.2121
PC	7.2634	1.5828	10.3836	1.2792	9.5222	1.6430	7.9276	1.5140

(b) Midbrain

Female:	CC		BC		CB		BB	
Biomarker	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
SOD	5.3958	0.2009	6.7881	0.4584	6.1202	0.4564	6.2948	0.2983
GPX	0.0902	0.0082	0.1144	0.0110	0.1042	0.0085	0.1091	0.0134
OXY	17.7458	1.3445	19.1167	1.3611	17.6180	1.2871	19.7754	1.7084
PC	12.6329	1.4267	8.2373	2.6900	10.2774	2.0570	10.0165	2.3946

Male:	CC		BC		CB		BB	
Biomarker	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
SOD	6.0460	0.4598	5.8433	0.2084	5.5059	0.4291	5.8367	0.3075
GPX	0.0067	0.0006	0.0074	0.0016	0.0085	0.0010	0.0091	0.0010
OXY	18.9335	1.0700	17.0323	0.9868	18.0475	1.3385	19.8249	2.0350
PC	8.9668	1.4793	7.7708	1.3903	10.3069	2.4990	7.6081	0.9878

(c) Cerebellum

Female:	CC		BC		CB		BB	
Biomarker	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
SOD	3.0723	0.1927	2.7341	0.1835	2.6855	0.2474	2.8901	0.2083
GPX	0.0773	0.0095	0.1158	0.0129	0.1087	0.0123	0.1117	0.0120
OXY	10.8112	0.5022	10.6479	0.9174	10.9963	0.8443	8.9539	0.7967
PC	11.5053	1.7998	10.9484	1.2433	10.7885	3.0476	8.5073	1.9590

Male:	CC		BC		CB		BB	
Biomarker	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
SOD	2.9475	0.1147	3.1109	0.2679	3.0227	0.1636	3.0173	0.1302
GPX	0.0888	0.0107	0.0915	0.0120	0.1053	0.0104	0.0940	0.0101
OXY	10.0170	0.7052	12.1001	1.3477	10.6748	0.8337	9.5611	0.8460
PC	12.6398	1.9469	11.8698	3.0155	14.3266	2.3671	11.4430	2.4430

Table 5.2 Results of the LME modelling of potential long-term effects of the pre- and post-hatching treatment (in the table referred as PRE and POST, respectively), sex, and their interactions on basal oxidative stress biomarkers (superoxide dismutase, SOD; glutathione peroxidase, GPX; non-enzymatic antioxidant capacity, OXY; and protein carbonyls, PC) in the adult Japanese quail. Outcomes in bold indicate the factors included in the final model; the other factors were subsequently excluded from the model as they were not significant ($p > 0.05$), although the effects of both pre- and post-hatching treatments were always maintained in the final model.

(a) Red blood cells				
Response variable	SOD	GPX	OXY	PC
PRE	$\mathbf{F_{1,62.19} = 1.70, p = 0.197}$	$\mathbf{F_{1,67.58} = 4.865, p = 0.031}$	$\mathbf{F_{1,69.37} = 2.038, p = 0.158}$	$\mathbf{F_{1,69.37} = 0.204, p = 0.653}$
POST	$\mathbf{F_{1,49.61} = 0.27, p = 0.609}$	$\mathbf{F_{1,54.62} = 5.468, p = 0.023}$	$\mathbf{F_{1,61.92} = 1.280, p = 0.262}$	$\mathbf{F_{1,69.09} = 0.011, p = 0.918}$
Sex	$F_{1,62.21} = 3.434, p = 0.069$	$F_{1,69.75} = 3.598, p = 0.062$	$F_{1,68.05} = 2.760, p = 0.101$	$\mathbf{F_{1,69.28} = 7.496, p = 0.008}$
PRE X SEX	$F_{1,50.85} = 0.098, p = 0.756$	$F_{1,62.25} = 0.991, p = 0.323$	$F_{1,65.63} = 0.830, p = 0.366$	$F_{1,68.10} = 0.310, p = 0.580$
POST X SEX	$F_{1,52.16} = 0.198, p = 0.658$	$F_{1,61.47} = 0.004, p = 0.947$	$F_{1,62.95} = 0.119, p = 0.732$	$F_{1,65.54} = 0.292, p = 0.591$
PRE X POST	$F_{1,51.72} = 0.118, p = 0.733$	$F_{1,60.86} = 0.671, p = 0.416$	$\mathbf{F_{1,65.50} = 5.753, p = 0.019}$	$F_{1,62.99} = 0.010, p = 0.922$
PRE X POST X SEX	$F_{1,53.10} < 0.0001, p = 0.986$	$F_{1,62.61} = 1.053, p = 0.309$	$F_{1,63.06} = 0.028, p = 0.869$	$F_{1,63.41} = 3.565, p = 0.064$
(b) Midbrain				
Response variable	SOD	GPX	OXY	PC
PRE	$\mathbf{F_{1,71.00} = 2.138, p = 0.148}$	$\mathbf{F_{1,70.97} = 0.697, p = 0.407}$	$\mathbf{F_{1,71.00} = 0.770, p = 0.383}$	$\mathbf{F_{1,68.29} = 1.925, p = 0.170}$
POST	$\mathbf{F_{1,71.00} = 0.215, p = 0.644}$	$\mathbf{F_{1,62.17} = 0.011, p = 0.917}$	$\mathbf{F_{1,71.00} = 0.104, p = 0.748}$	$\mathbf{F_{1,64.56} = 1.117, p = 0.734}$
Sex	$F_{1,70.00} = 0.854, p = 0.360$	$F_{1,68.22} = 0.032, p = 0.858$	$F_{1,70.00} = 0.029, p = 0.866$	$F_{1,66.72} = 1.538, p = 0.219$
PRE X SEX	$F_{1,69.00} = 1.592, p = 0.211$	$F_{1,67.05} = 0.769, p = 0.384$	$F_{1,68.00} = 0.698, p = 0.406$	$F_{1,65.78} = 0.682, p = 0.412$
POST X SEX	$F_{1,68.00} = 0.662, p = 0.419$	$F_{1,65.03} = 0.265, p = 0.608$	$F_{1,67.00} = 0.058, p = 0.810$	$F_{1,62.859} = 0.409, p = 0.525$
PRE X POST	$F_{1,67.00} = 0.320, p = 0.574$	$F_{1,62.24} = 0.303, p = 0.584$	$F_{1,69.00} = 1.146, p = 0.288$	$F_{1,63.56} = 0.577, p = 0.450$
PRE X POST X SEX	$F_{1,66.00} = 2.247, p = 0.139$	$F_{1,63.01} = 0.486, p = 0.488$	$F_{1,66.00} = 0.483, p = 0.490$	$F_{1,63.15} = 0.888, p = 0.350$
(c) Cerebellum				
Response variable	SOD	GPX	OXY	PC
PRE	$\mathbf{F_{1,70.12} = 0.005, p = 0.943}$	$\mathbf{F_{1,70.43} = 1.106, p = 0.297}$	$\mathbf{F_{1,70.00.} = 0.292, p = 0.591}$	$\mathbf{F_{1,70.85} = 0.982, p = 0.325}$
POST	$\mathbf{F_{1,59.70} = 1.120, p = 0.731}$	$\mathbf{F_{1,63.84} = 3.567, p = 0.063}$	$\mathbf{F_{1,61.92} = 1.700, p = 0.197}$	$\mathbf{F_{1,68.73} = 0.014, p = 0.905}$
Sex	$F_{1,69.57} = 1.356, p = 0.248$	$F_{1,69.19} = 0.907, p = 0.344$	$F_{1,66.96} = 0.019, p = 0.890$	$F_{1,69.74} = 1.530, p = 0.220$
PRE X SEX	$F_{1,67.64} = 0.369, p = 0.545$	$F_{1,68.00} = 2.043, p = 0.158$	$F_{1,65.93} = 1.948, p = 0.167$	$F_{1,66.82} < 0.0001, p = 0.991$
POST X SEX	$F_{1,61.68} = 0.269, p = 0.606$	$F_{1,67.00} = 0.044, p = 0.834$	$F_{1,64.00} = 0.011, p = 0.918$	$F_{1,68.32} = 0.521, p = 0.473$
PRE X POST	$F_{1,61.54} = 0.392, p = 0.534$	$F_{1,63.33} = 2.721, p = 0.104$	$\mathbf{F_{1,66.20} = 4.428, p = 0.039}$	$F_{1,66.71} = 0.324, p = 0.571$
PRE X POST X SEX	$F_{1,64.00} = 1.760, p = 0.189$	$F_{1,66.00} = 0.223, p = 0.639$	$F_{1,63.07} = 0.391, p = 0.534$	$F_{1,65.34} = 0.017, p = 0.897$

5.5 Discussion

The results of the present study reinforce the idea that overexposure to stress hormones in early life causes long-term changes in the cellular redox status. This study represents the first experimental evidence that the magnitude of these changes is also dependent upon interactions across different developmental stages. The results also suggest that some tissues may be more sensitive to the long-term effects of glucocorticoid programming, with important implications for the design of future studies as well as the potential long-term effects of early life stress on adult phenotypes.

Developmental B had an impact on the blood redox physiology, with both the pre- and post-hatching B-exposed individuals showing on average elevated activity of the antioxidant enzyme GPX compared to the controls. The magnitude of this effect was markedly larger in the individuals that experienced the combined pre- and post-hatching B treatments, with a 50% higher GPX activity than the controls. Blood non-enzymatic antioxidant capacity (OXY) decreased in the pre- or post-hatching B-treated birds compared with the controls, but conversely, such a decrease was less pronounced in the birds that were exposed to the combined pre- and post-hatching B treatments. Altogether, these results suggest that matching pre- and post-hatching stressful developmental environments triggered both additive and interactive responses in the developing individuals, which gave rise to a distinct oxidant challenge compared with that induced in the pre- or post-hatching glucocorticoid-exposed birds. Erythrocytes are among the most abundant circulatory cells in the vertebrate organism and their antioxidant system provides protection not only to themselves but also to other tissues and organs (Pandey *et al.*, 2011). The lack of treatment differences on protein carbonyls, a biomarker of oxidative damage, suggests that the up-regulation of GPX in the pre- and post-hatching B-treated birds may be an adaptive compensatory strategy to enhance resistance to reactive oxygen species-damage in the body. Clearly, the burden of reactive oxygen species is counteracted by a complex antioxidant defence system (Pamplona and Costantini, 2011). As I found no treatment effect on SOD, the first line of antioxidant defence, it is likely that developmental B altered the secondary rather than primary oxidant-antioxidant signalling pathways (Figure

5.1). I interpreted the observed trends towards the reduction of OXY across the B-treated individuals as a consequence of the increased GPX activity. In fact, GPX uses the reduced glutathione to detoxify the cell from hydrogen peroxide derived from SOD activity, but also from early peroxidation compounds (i.e. hydroperoxides) continuously produced in the cell and known precursors of end-products of oxidative damage (Halliwell and Gutteridge, 2007). I, therefore, propose that the developmental B-exposed birds, especially those that experienced the combined B protocols, were challenged with higher production of these intermediate damaging compounds, which were efficiently quenched by prioritising up-regulation of GPX. Future work integrating other measures, specifically glutathione, will be needed to further validate this possibility. Nevertheless, such a hypothesis finds support from a recent study in the chicken (*Gallus gallus*) in which *in ovo* overexposure to B has been found to elevate baseline plasma hydroperoxides in 3-week-old juveniles (Haussmann *et al.*, 2012). However, in the latter study no post-hatching manipulations were undertaken. The results of the present study clearly indicate that post-hatching cues during development do occur and do interact with the effects of previous pre-hatching stimuli. Such effects may be interpreted as potential adaptive regulatory responses occurring during the individual's development. These results suggest that the plasticity/propensity of the redox system to glucocorticoid-induced change differs among the developmental phases, possibly depending on the maturation of the antioxidant defences (Surai, 2002; Spicer and Burggren, 2003). Further studies *in ovo* and early post-hatching are needed to test this possibility.

The magnitude of the effects of the developmental B manipulation on brain oxidative balance was lower than that predicted and, importantly, differed between brain tissue types. In fact, treatment differences were observed in the cerebellum but not in the midbrain samples, suggesting that the developmental treatment, which was designed to increase pre- and post-hatching glucocorticoid exposure within the relevant biological ranges, induced brain local-specific changes rather than a general unified effect. Brain region-specific effects on antioxidants have also been reported in juvenile rats that experienced adverse events during neonatal life (maternal separation, diet manipulation, and handling) (Marcolin *et al.*, 2012; Uysal *et al.*, 2012). Although cerebellum GPX,

similarly to the response in the erythrocytes, tended to be higher in the B-treated birds compared with the controls, the magnitude of this up-regulation was relatively low (around 25%), clearly limiting statistical power. However, as the brain contains low concentrations of antioxidants compared with other body tissues (Halliwell and Gutteridge, 1985), the observed increase in GPX activity may be biologically important. It should be noted that the BB birds showed consistent antioxidant patterns in the cerebellum as seen in the erythrocytes, with higher GPX activity and lower OXY. These data reinforce the idea that such individuals were more challenged by pro-oxidants than the adult birds that had experienced elevated exogenous B only as embryos (BC) or as juveniles (CB). Taken together, these data suggest that prolonged stressful experiences across developmental stages can produce stronger signalling effects on secondary antioxidant pathways, not only in the blood circulation, but also in specific post-mitotic neuronal tissues. BC or CB birds could maintain high levels of cerebellar OXY probably because of remobilisation of thiols and other dietary-derived antioxidants from other tissues through the blood circulation (Dass *et al.,* 1992). This would suggest competition among tissues for antioxidant protection that could be solved by prioritising tissues whose functions are key in specific developmental windows or are more vulnerable to oxidative stress. Although it was beyond the scope of this work, further studies may wish to test this "*tissue competition hypothesis*" by extending oxidative stress measures to other tissues, including heart, spleen and liver. The cerebellum is a vital brain structure controlling motor skills and cognition, and increased protein carbonylation in this area is often linked with loss of such abilities (Forster *et al.,* 1996; Manda *et al.,* 2008). Therefore, it is plausible that the alterations observed in the antioxidant capacity in the BB birds may have been activated as a defence mechanism to avoid rises in cerebellum protein carbonyls, thereby protecting impairment in cognitive abilities. On the other hand, since elevated developmental glucocorticoids can markedly reduce long-term individual's lifespan (Monaghan *et al.,* 2012), probably via increasing the rates of telomere shortening (Haussmann *et al.,* 2012), it is also possible that such changes in the antioxidant defences over a longer term may impinge on neuronal integrity and enhance vulnerability to neurodegenerative diseases (Ramassamy *et al.,* 1999; Schuessel *et al.,* 2004).

I do not know the underlying mechanisms that may explain the observed region-specific actions of glucocorticoids in the brain. Among the variety of factors involved, such as the magnitude and timing of glucocorticoid overexposure, the distribution of brain corticosteroid receptors (i.e. MR and GR) is likely to play an important role (You *et al.*, 2009). Across vertebrate species, corticosteroid receptors in the brain are more concentrated in areas such as the hippocampus, hypothalamus, amygdala and the cerebellum (e.g. Kovacs *et al.*, 1989; Patel *et al.*, 2000; Dickens *et al.*, 2009), and their densities may differ even across brain nuclei. In fact, the transcriptome analysis described in Chapter 4 clearly showed marked differences in the overall gene expression patterns between hippocampus and hypothalamus, and such differences also included the expression of both MR and GR. Furthermore, a recent study in young zebra finches (*Taeniopygia guttata*) demonstrated that the labelling intensities of GR immunoreactive neurones differed in the nuclei located in the telencephalon and diencephalon (Shahbazi *et al.*, 2011). As the midbrain punches also contained telencephalic and diencephalic nuclei, I am not able to exclude the possibility that unequal amounts of corticosteroid receptors between these two brain regions may have diluted out the effects of elevated exogenous B on the antioxidant pathways as seen in the cerebellum. Further studies looking at the effects of glucocorticoids within specific neuronal nuclei and across different brain areas will be needed in order to test the biological relevance of this hypothesis.

5.6 Conclusion

In conclusion, this study shows that interactions between environmental conditions during key developmental stages can shape adult oxidative status through the action of glucocorticoids. These results reinforce the importance of early post-natal/hatching interventions as a mechanism to manipulate previous pre-natal/pre-hatching phenotypic adjustments (Vickers *et al.*, 2005). Overall, my data suggest that prolonged stressful experiences during pre- and post-hatching development can produce interactive effects that result in changes in the antioxidant defences in the blood and in post-mitotic neuronal tissues,

depending on the brain region. These long-term shifts in the basal antioxidant defences may represent adaptive phenotypic adjustments to efficiently prevent oxidative damage to biomolecules, but leave open the possibility that any potential long-term consequences affecting cellular senescence may arise through high investment in antioxidant protection. This study is relevant to both biomedical researchers and evolutionary ecologists attempting to probe the underlying mechanisms linking stress hormones and oxidative status changes in an early development framework, and how they may be potentially associated with health and long-term survival.

6. Chapter
General Discussion

6.1 Review of the findings

The main aim of this thesis was to investigate how and the extent to which, physiological overexposure to glucocorticoid stress hormones during pre- and post-hatching development influences an individual's phenotypic trajectories that may persist throughout life, using the Japanese quail as the study species. This avian model provided me with the opportunity to easily manipulate both pre- and post-hatching environmental conditions, removing the confounding factors caused by the physiological intimacy between mother and offspring that is present in other vertebrate taxa, such as mammals (Spencer *et al.*, 2009; Henriksen *et al.*, 2011).

I first performed an experiment that allowed me to investigate the potential changes in adrenocortical activity in response to a standardised environmental stressor presented during the linear phase of post-hatching growth, at day 8 and day 16 (Chapter 2). The main results from this study suggested that the magnitude of the acute stress response declined with age, the same directional trend that has been previously found in the other few studied precocial birds (e.g. Holmes *et al.*, 1989; Dickens and Romero, 2010). Interestingly, the opposite directional changes, an increase of adrenocortical activity in response to acute stress, have been reported in altricial birds (e.g. Sims and Holberton, 2000; Love *et al.*, 2003; Walker *et al.*, 2005; Wada *et al.*, 2007). Altogether, these results supported the "*Developmental Hypothesis*" (e.g. Schwabl, 1999) and warrant further investigations.

In the main experiment of this project (Chapter 3, 4, and 5), I exposed the embryos to exogenous corticosterone (B, the main avian glucocorticoid), via egg-injections directly into the yolk, and/or the juveniles (from post-hatching days 5-19), via oral hormone supplementation using B-injected mealworms. At both developmental stages, the exogenous B doses were within the physiological

ranges of the study species. The pre-hatching treatment, designed to simulate maternal transfer of B into the egg, may be of great adaptive importance as it is believed to 'physiologically prime" the embryo to survive in an environment that may be potentially stressful following hatching (e.g. Saino *et al.*, 2005; Hayward *et al.*, 2006), whilst the post-hatching treatment mimicked prolonged stressful environmental conditions (Spencer *et al.*, 2009) in early post-hatching development. The three stress-phenotypes (i.e. BC, CB, and BB birds) allowed me to disentangle physiological and global gene expression responses that occurred as a consequence of pre-hatching exposure to B, post-hatching exposure to B or a combination of both the treatments. I showed that both pre- and post-hatching B induced changes in the Hypothalamic-Pituitary-Adrenal axis (HPA axis) responsiveness and circulating energy sources (glucose and triglycerides) in the blood (Chapter 3). The main results suggested that the effects of post-hatching B on the activity of the HPA axis and blood biochemistry were predominantly over the short-term (post-hatching day 22) and these effects were sex-dependent. Specifically, the juvenile females that experienced post-hatching B, regardless of the previous pre-hatching experiences, showed shorter stress responses in comparison with the other treatment groups. Post-hatching B also caused significant changes in basal triglycerides, which interacted with sex and basal glucose concentrations. In contrast, the effects of pre-hatching B on the stress physiology were mainly evident over the long-term in the adults (post-hatching day 64). In fact, the adult birds previously stressed *in ovo* exhibited higher B concentrations over the stress response than control birds. Interestingly, this effect was not evident in the birds that had been subjected to the combined treatments. Also, the birds that experienced pre-hatching B had reversed basal sex-specific glucose concentrations compared to the other treatment groups. Although to the best of my knowledge, this is the first study that have attempted to analyse the effects of elevated glucocorticoids on glucose and lipid concentrations in the blood, there are previous studies in birds that have examined the effects of early life stressors on HPA axis activity. The results from these studies showed different outcomes even within the same species (Chapter 3, Section 3.5; see also reviewed by Henriksen *et al.*, 2011). In this regard, a very recent study in the Japanese quail using the same pre-hatching treatment as in the present study but a different

post-hatching stressful protocol (unpredictable food regime), found opposite directional changes in the B stress responses compared to this study, with the pre-hatching injected birds showing truncated stress responses when 42-44 days of age than the pre-hatching controls (Zimmer *et al.*, 2013). Therefore other than the actual hormonal dose, other factors including husbandry and housing conditions, handling for morphological measurements, and the type of post-hatching conditioning imposed on the animals might be very important and differences in experimental designs across studies need to be carefully evaluated in the comparative approach.

Chapter 4 focused on the influences of elevated exposure to B during pre- and post-hatching development on the transcriptome signature in two target brain structures controlling the HPA axis, the hippocampus and hypothalamus, of the adult quail. The main findings suggested that early life stress induced distinct tissue-specific modifications in global gene expression patterns. The number of genes that were differentially expressed was not large, a finding that was consistent with previous studies that examined the effects of variations in early life conditions in other species (Weaver *et al.,* 2006; Lindqvist *et al.*, 2007; Nätt *et al.*, 2009; Goerlich *et al.*, 2012). The significantly altered gene expression patterns involved well known key candidate genes in the regulation of the HPA axis, such as the mineralocorticoid receptor (MR), vasotonergic system, brain-derived neurotrophic factor, and serotonin receptors. The study also suggested important novel regulatory mechanisms/pathways that appeared to be modified by pre- and post-hatching B exposure into adulthood, such as those regulating oxidation processes. Importantly, the analysis showed that there were distinct tissue-specific cumulative, as well as some opposite effects, on the brain transcriptome signature induced by the interactions between pre- and post-hatching B treatments.

Chapter 5 focused on the effects of pre- and post-hatching B on body oxidative stress in adult birds. This was assessed by looking at both non-enzymatic (total non-enzymatic antioxidant capacity) and enzymatic antioxidant defences (superoxide dismutase and glutathione peroxidise) as well as oxidative damage (protein carbonyls) in key target tissues, the red blood cells and the brain (cerebellum and midbrain). The main results showed that the effects of the B

treatments produced specific modifications in the secondary line of antioxidant defence pathways in the erythrocytes and cerebellum tissues (glutathione peroxidise and non-enzymatic antioxidant capacity), but no effects were observed in the midbrain regions. The magnitude of the significant differences on the antioxidant defences depended upon interactions between pre- and post-hatching stimuli. I also found differences among tissues with stronger effects in the blood than in the cerebellum.

Overall, the general theme of this thesis suggested that development is a very complex phenomenon, encompassing dynamic changes in the physiological stress responses that are likely to be linked with the species' life histories and developmental strategies. Importantly, both pre- and post-hatching exposure to B can have the potential to "re-set" individuals' phenotypic trajectories. The multidisciplinary approach undertaken in this work highlighted the complexity of these phenotypic responses as they appeared to be tissue specific, with alterations at both the physiological and the gene expression level. One of the main questions arising after the overview of the main findings is: are these changes ecologically important? Specifically, can early life stress affect Darwinian fitness and survival?

6.2 Developmental plasticity and early life stress: an evolutionary perspective

The data presented in Chapter 2 supported the "Developmental Hypothesis" as mentioned above (Section 6.1). The main principle of such hypothesis is the co-evolution of species' developmental strategies and the hormonal signalling pathways. The stress response becomes demonstrable much earlier in the life of precocial birds (at least from the later stages of pre-hatching development) compared to altricial species that show adult-like stress responses close to or at fledgling (reviewed by Wada, 2008). From an evolutionary perspective, these differences may be explained by variations in the developmental mode between precocial and altricial species (see Chapter 2). It would be interesting, however, to appraise the biological variability in the maturation of the HPA axis system in

birds that have evolved semi-altricial/semi-precocial developmental strategies and examine if, and how, they fit within the comparative framework of the available literature. Ideally, such experiments should be conducted in the wild and would require careful considerations of the specific time of sampling, which should take into account ecologically relevant developmental windows (e.g. hatching, fledgling, nutritional independence) and the variation in parental care across specific nestling stages. We also lack studies in the natural context that have examined the variation of the juvenile stress responses within populations of the same species. It is plausible that the development of stress physiology may vary depending on the environment, such as different degrees of predator densities. Again, this hormonal phenotypic plasticity would be expected to be dependent on the developmental mode. Under this scenario, future studies in juveniles of bird species adapted to cope with extreme environments, such as desert and high latitude, will be extremely important for comparative research with the scope to assess the evolutionary meaning for variation in the ontogeny of the stress responses across species.

Whether the effects of early life stress on the phenotype are adaptive because they convey ecologically relevant information on the current/future environmental conditions to the growing individuals or maladaptive physiological constraints that negatively affect Darwinian fitness has been hotly debated (Henriksen *et al.*, 2011, Schoech *et al.*, 2011; Love *et al.*, 2013). The predominant idea is that phenotypic plasticity, driven by early life experiences, exerts adaptive responses largely over the short-term, but may have physiological costs later in adult life (Gluckman *et al.*, 2007). However, new theories, supported by some empirical work are emerging and have emphasised the importance of testing predictions on the potential adaptive value of the early environment in different post-natal/hatching environmental contexts and across multiple individual life stages (Monaghan, 2008). Although the main experiment of this thesis was not specifically designed to test adaptive or non-adaptive predictions of early life stress, some overall conclusions may be drawn by interpreting the phenotypic and genomic results together. For example, the physiological effects of post-hatching B were mainly visible over the short-term and induced sex-specific changes on the dynamics of the stress response, basal triglycerides and basal glucose concentrations (see Chapter 3). These results

suggested that the post-hatching B-treated quail can adapt to the current prevailing stressful environment with immediate physiological changes that "re-set" the regulation of the stress response as well as the allocation of available energy resources in the bloodstream. Similar condition-driven adrenocortical plasticity has been described in previous work in young free-living European Starlings experiencing poor quality maternal care and has been termed the "*Reactive Adaptive Response*" (Love and Williams, 2008). The data reported here (Chapter 3) suggested that these immediate/short-term responses cause changes not only to the activity of the HPA axis, but also to other important physiological mechanisms involved in energy transport and lipid deposition. Furthermore, in the study conducted by Love and Williams (2008) the pre-hatching programming cues of B constrained the degree of post-hatching reactive plasticity in the responsiveness of the HPA axis of the juvenile birds. In contrast, in this study post-hatching B-mediated effects on the young quail did not interact with the pre-hatching experiences. This suggested that post-hatching environmental cues are of primary importance in quail. Clearly, precocial birds reach nutritional independence soon after hatching and rely less on maternal care than altricial birds. Therefore, they need to adapt quickly to unpredictable environmental conditions and the evolution of a hormonal signalling system that is highly sensitive to the immediate post-hatching cues may be a better strategy than relying on previous maternal predictions (assuming that elevated yolk B is a key coding signal integrated by the embryo). In this regard, glucocorticoids may be very important as they can enhance fear, mobility and vigilance behaviours, so allowing the juveniles to better avoid predators and reduce risk-taking behaviours (Breuner *et al.*, 1998a, b; Janczak *et al.*, 2006).

Intriguingly, the physiological effects of pre-hatching B on the activity of the HPA axis and energy metabolism were visible predominantly during adulthood and they appeared dependent on early post-hatching conditions (Chapter 3). An overview of these results, including those observed on cellular redox balance (Chapter 5), suggested that matching pre- and post-hatching B triggered interactive long-term effects on the pre-hatching glucocorticoid-exposed phenotypes when adults. A variety of long-term context-dependent responses were observed in the pre- and post-hatching B-treated birds. Specifically, a post-

natal stressful environment had on some occasions intensified (e.g. effects on red blood cell glutathione peroxidise, Chapter 5), or mitigated/buffered (e.g. results on the HPA axis responsiveness shown in Chapter 3) the effects of previous embryonic exposure to B. But what may be the mechanism mediating this developmental-glucocorticoid-dependent physiological plasticity?

Corticosteroid receptors (mineralocorticoid and glucocorticoid receptors, MR and GR respectively) act as transcription factors and are well known to be actively involved in regulation of the stress responsiveness and several stress-related behaviours (de Kloet *et al.*, 2005a, b; Joel *et al.*, 2008). As mentioned in more detail elsewhere (Chapter 1), glucocorticoids have an affinity to MR 5-10 times higher than GR and, therefore, MR remain tonically activated by basal glucocorticoid levels. As a result of the different affinity of MR and GR in binding glucocorticoids, the balance of expression of both MR and GR is thought to be critical to maintain homeostasis within an organism (Reul and de Kloet, 1985; de Kloet *et al.*, 2005a). Previous studies in birds have shown that both MR and GR receptors in the brain can be affected by early life stressful conditions (Banerjee *et al.*, 2012) or chronic exposure to stress (Dickens *et al.*, 2009), similar to what has been observed in many mammalian studies (reviewed by Oitzl *et al.*, 2010). Here, I found a higher expression of the gene coding MR (NR3C2) in the hippocampus of the adult B-exposed phenotypes compared to the controls (Chapter 4). While hippocampal GR transcript levels (NR3C1) did not differ across the treatment groups, the analysis of the balance between MR:GR expression abundances showed different trends across the treatments, with the adult quail that experienced stress during both pre- and post-hatching developmental periods having lower MR/GR ratio compared to the pre- or post-hatching B-treated birds (Chapter 4). I propose that this different hippocampal MR: GR ratio in the adult pre- and post-hatching B-exposed birds might be an important regulatory mechanism to explain the interactions between pre- and post-hatching B both at the physiological (Chapter 3 and 5) and gene expression level (Chapter 4). However, as the transcriptional analysis was limited to pooled RNA samples, investigations at the individual level on MR and GR would be a future important step to experimentally validate this hypothesis. Ideally, these further investigations should examine both gene expression levels and protein content of corticosteroid receptors as these measurements may not necessarily

correlate between each other (e.g. Ahmed *et al.*, *in press*). In the awareness of the high variations across the results in the area of developmental stress (e.g. Henriksen *et al.*, 2011), it would be important to test this hypothesis on multiple post-hatching scenarios, especially under stressful and non stressful conditions, and across different species in order to sample the variability underlying the regulatory mechanisms in the brain associated with early life stress. Also, in this study, the modifications across treatments observed in MR gene expression were shown to be functionally linked to several other genes, such as those coding neurotrophic factors, neuronal oxidation processes, and other neurohormones. Therefore, it is likely that developmental exposure to glucocorticoids exerted changes in an array of inter-connected transcriptional pathways rather than on the expression of single candidate genes (Chapter 4). In fact, the data presented in Chapter 4 suggest that such transcriptional pathways are likely to be linked with neurotrophin factors (hippocampal BDNF expression was increased in all the B-treated birds compared to the control birds) as well as the serotonergic system (3 hypothalamic serotonin receptors, HTR3A, HTR2C, and HTR1D, were all up-regulated in the birds treated with B post-hatching compared to the post-hatching control birds). Another important possible route by which developmental stress can induce gene expression changes is via epigenetic processes, such as DNA methylation and histone modifications (Murgatroyd *et al.*, 2009; Weaver *et al.*, 2004). For example, childhood adversities have been shown to increase CpG methylation of the GR promoter in human leukocytes (Tyrka *et al.*, 2012) and the methylation of the corticotrophin releasing hormone (CRH) promoter in different brain areas in rats (Sterrenburg *et al.*, 2011). Interestingly, enhanced CpG methylation of both the GR and CRH promoters have been observed recently in the hypothalamus of adult chickens exposed to B pre-hatching in comparison with adult controls, providing evidence that such changes can be attributed to the direct effects of B exposure itself (Ahmed *et al.*, *in press*). Taken together these data open the question of possible enduring trans-generational effects of developmental stress via epigenetic mechanisms and future research should attempt to integrate gene expression analysis with DNA methylation measurements. But can the combined effects of stress during the pre- and post-natal/hatching periods result in a better adapted adult phenotype compared to that induced by pre- or post-natal/hatching stress on

their own? At this stage, this remains unclear. The functional analysis pointed to a series of negative cumulative effects associated with the combined early life stressful treatments, such as cancer, neurological diseases, and hereditary disorders. However, the available literature in genome-wide analyses is highly biased by research in the biomedical field, which rarely consider the potential adaptive benefits of developmental stress in later life. We do have several line of evidence across studies in mammals and birds showing that developmental stress can exert long-term changes on an array of behaviours, including exploratory behaviours in novel environments, neophobia, song, memory and spatial learning, and aggressive responses (e.g. reviews by Henriksen *et al.*, 2011; Schoech *et al.*, 2011). Some of these effects do not appear to be simply unavoidable physiological developmental constraints, but rather adaptive responses that prepare the individual to adopt appropriate behavioural responses in environments in which stressors may be frequently encountered (Meylan and Clobert, 2005; Zimmer *et al.*, 2013; see also review by Love *et al.*, 2013). In the present thesis, the data on the redox physiology concur to provide, at least in part, some support for the hypothesis of adaptive advantages of developmental stress programming. In fact, the analysis of redox oxidative balance in the blood of the adult quail clearly showed that the activity of glutathione peroxidise in the erythrocytes, an important vehicle for the transport of antioxidants in the body, was 50% higher in the BB birds than in the CC birds, suggesting that the combined action of pre- and post-hatching B may have triggered cumulative long-term adaptive protective responses in the antioxidant system of these birds. Moreover, as I found no significant increases of protein damage in any of the tissues examined, it seems likely that the BB birds could avoid a condition of oxidative stress potentially via the observed alterations in antioxidant defences. On the other hand, as overexposure to post-hatching B has the potential to significantly reduce life expectancy in adulthood (Monaghan *et al.*, 2012) and embryonic exposure to stress hormones can accelerate telomere loss in red blood cells (Haussmann *et al.*, 2012), it is also possible that the phenotypic modifications in the BB quail may actually have negative effects on later fitness and/or survival. To the best of my knowledge, this is the first evidence showing that oxidative balance may be plastic to both pre- and post-hatching environmental cues and I do hope that it will encourage

future research in this area. For future research, it will be extremely important to perform longitudinal studies in short-lived animal models, including the Japanese quail, and track changes on reproductive success, survival and ageing trajectories across the multiple stage of adult life. These data are fundamental to provide a framework to interpret from an evolutionary perspective fitness costs and benefits of the physiological changes associated with developmental stress programming. We also need more experimental work to examine whether the effects of developmental stress can be transmitted/extended to the following generations and to elucidate the underlying mechanisms of actions mediating such inheritance (likely associated with changes in the epigenome). These studies are critical to fully understand the complicated interplay among developmental stress, Darwinian fitness and survival. The study of such interplay may also help to reconcile the well known paradigm “nature *vs* nurture” and explore in more depth the relationships between development and evolutionary processes.

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Appendix

Table A1. TopHat arguments that were deviated from the default settings in the final alignment of the RNA-seq quail reads to the chicken reference genome. Full detail regarding the alignment is presented in Section 4.3.10.

TopHat parameters	Setting used
--initial-read-mismatches (i.e. number of mismatches allowed for each read)	3 (default 2)
--segment-length (i.e. minimum segment read length)	18 (default 25)
--segment-mismatches (i.e. number of mismatches allowed in each segment alignment)	1 (default 2)
--min-anchor-length (i.e. number of bases supporting every junction involved in sliced alignments by at least one read)	12 (default 8)

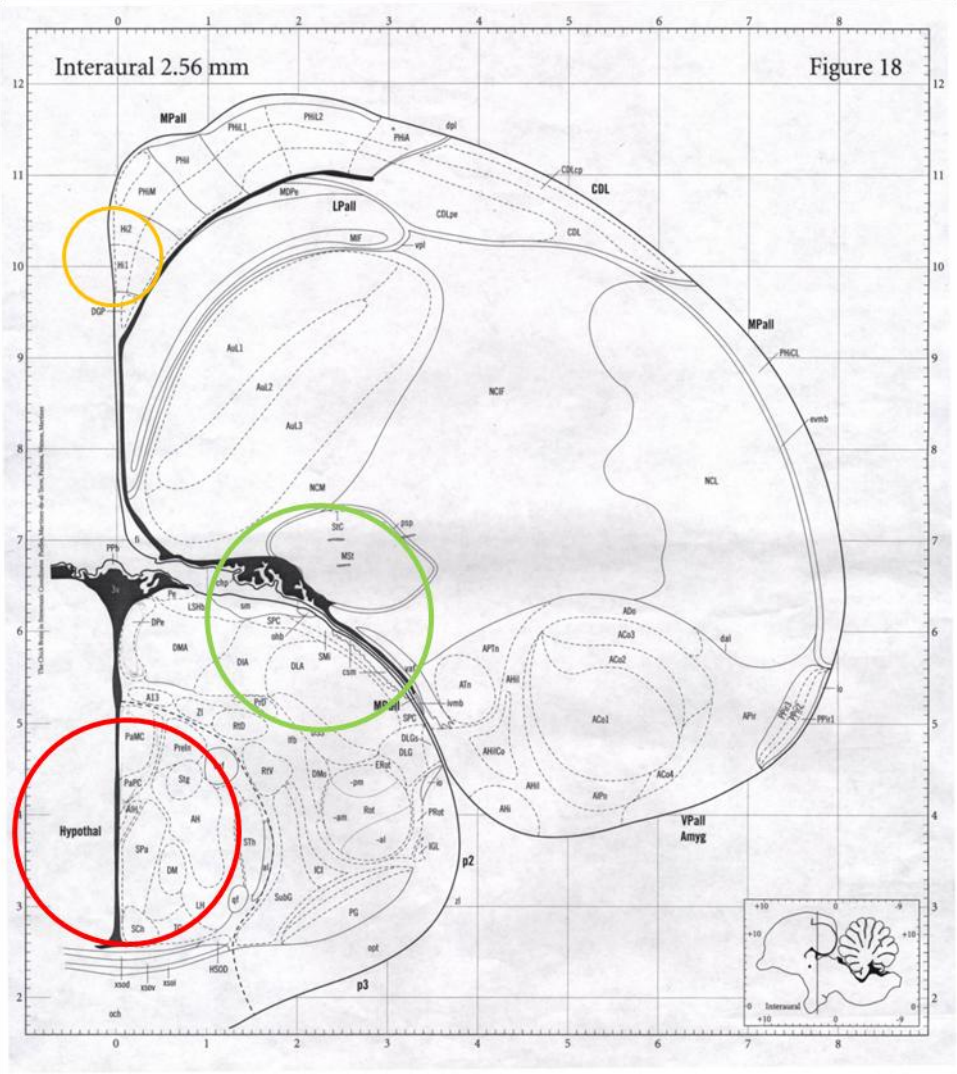

Figure A1 Schematic of one of the coronal brain section (interaural 2.56mm, Fig. 18 from the chicken brain atlas by Puelles *et al*., 2007) used a reference for obtaining the hippocampal (in yellow), hypothalamic (in red) and midbrain (in green) punches from the 2-mm-tick coronal sections of the quail brains. Hippocampal and midbrain punches were taken bilaterally; hippocampal and hypothalamic punches were used for the study presented in Chapter 4 while the midbrain punches were used for the study presented in Chapter 5. The size of each hippocampal punch was of 1mm diameter, whereas the size of each hypothalamic and midbrain punch was 2mm diameter.

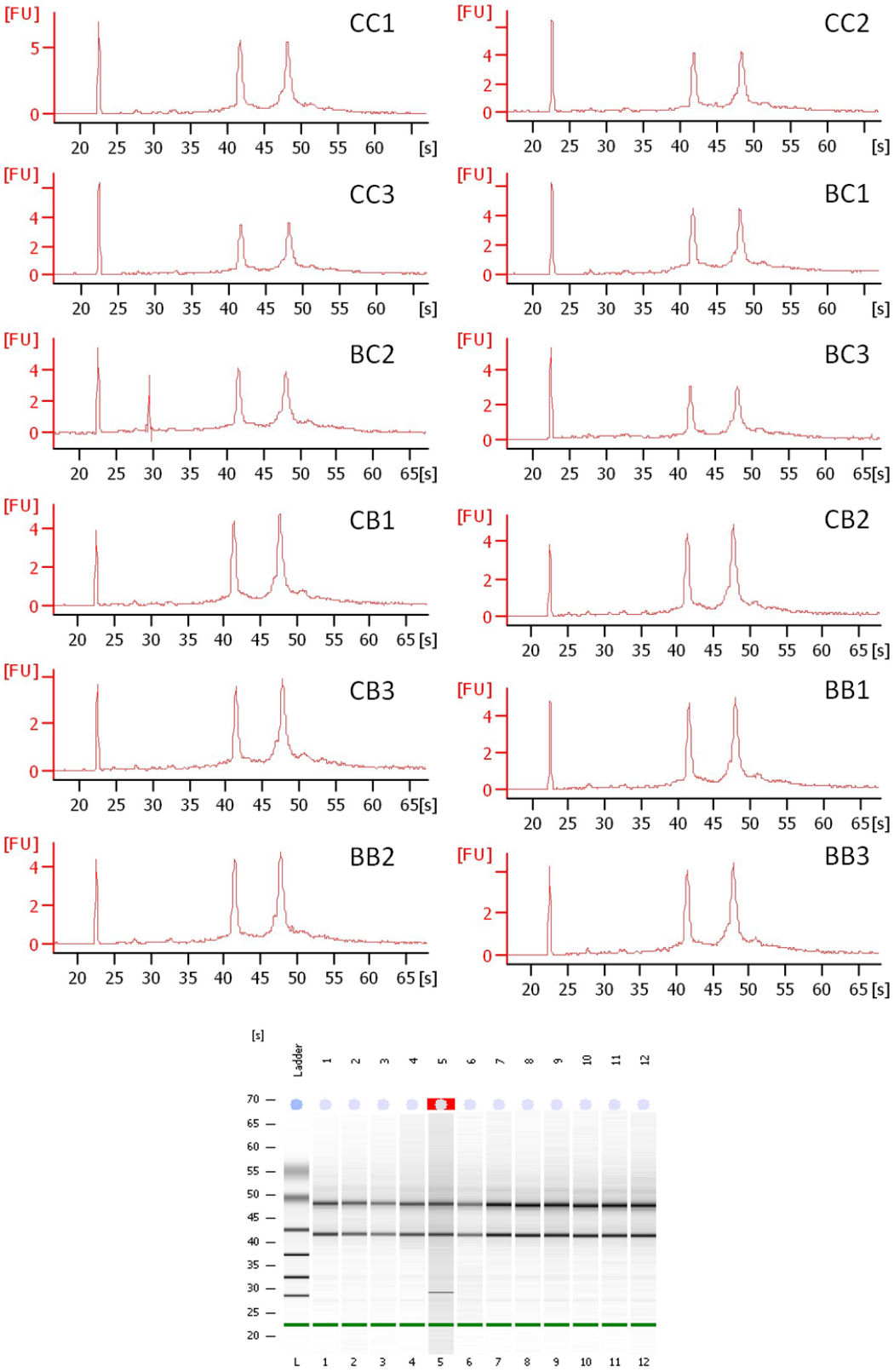

Figure A2. RNA quality control of the hippocampal RNA pooled samples assessed prior the start of the microarray experiments. In each graph is indicated the treatment group (CC, BC, CB or BB) with the numbers (1, 2, 3) representing the biological replicate in each treatment group.

Table A2. Ensembl Identifiers (Ensembl ID) and functional description of the genes with FRD ≤ 0.20 in the Bayseq models (DE); # indicates the row number in the inputted dataset.

DE2

Ensembl ID	#	Description
ENSGALG00000023456	16295	Homeobox CMIX
ENSGALG00000004239	3115	interferon induced transmembrane protein 5
ENSGALG00000006051	4537	cell division cycle 7-related protein kinase [Homo sapiens]
ENSGALG00000021242	14436	phosphohistidine phosphatase 1
ENSGALG00000013268	10001	Novel
ENSGALG00000016221	12114	EF-hand domain-containing family member
ENSGALG00000017405	13056	nitrogen permease regulator-like 3 (S. cerevisiae)
ENSGALG00000014773	10946	erbb2 interacting protein
ENSGALG00000004467	3295	CAP-Gly domain-containing linker protein 1
ENSGALG00000010928	8299	bone sialoprotein II
ENSGALG00000022843	15690	Novel

DE4

Ensembl ID	#	Description
ENSGALG00000013362	10050	calcium binding protein 7
ENSGALG00000016884	12655	solute carrier family 15 (oligopeptide transporter), member
ENSGALG00000015205	11302	tyrosinase-related protein-1
ENSGALG00000006726	5079	GATA binding protein 3
ENSGALG00000005978	4472	retinol binding protein 3, interstitial
ENSGALG00000011813	8954	HEG homolog 1 (zebrafish)
ENSGALG00000010718	8142	thrombospondin, type I, domain containing 7A
ENSGALG00000016095	12014	homeobox protein EMX1
ENSGALG00000011236	8532	brain-enriched guanylate kinase-associated homolog (rat)

DE6

Ensembl ID	#	Description
ENSGALG00000001807	1272	Homeobox
ENSGALG00000013155	9924	Novel
ENSGALG00000011424	8677	Eomesodermin

DE7

Ensembl ID	#	Description
ENSGALG00000023973	16812	alpha-1 collagen (I), partial
ENSGALG00000013294	10017	cytochrome P450, family 19, subfamily A, polypeptide 1
ENSGALG00000003541	2585	solute carrier family 32 (GABA vesicular transporter),

ENSGALG00000012544	9517	UDP-N-acetyl-alpha-D-galactosamine:polypeptide
ENSGALG00000003895	2853	PR domain containing 12
ENSGALG00000015419	11478	Proenkephalin
ENSGALG00000023913	16752	urocortin 3 (stresscopin)
ENSGALG00000013890	10383	melanocortin 5-receptor
ENSGALG00000008883	6745	transcription factor 7-like 2 (T-cell specific, HMG-box)

DE5

Ensembl ID	#	Description
ENSGALG00000000168	91	Adenosine receptor A1
ENSGALG00000008940	6789	Novel

Table A3. Normalised counts across the 3 biological replicates in each treatment group (CC, BC, CB and BB) of the genes with FDR ≤ 0.20 among the Bayseq models (DE2, DE4, DE5, DE6 and DE7) in the (a) hippocampus and (b) hypothalamus; # indicates the row number in the original dataset.

(a) Hippocampus

DE2	CC			BC			CB			BB			
#	1	2	3	1	2	3	1	2	3	1	2	3	FDR
16295	0	0	0	0	0	0	0	0	0	6	0	2	0.078
3115	5	2	4	2	2	8	1	3	5	3	2	152	0.081
4537	5	6	1	4	5	5	3	5	4	31	5	23	0.099
14436	302	313	333	341	312	328	319	309	289	443	364	430	0.112
10001	384	336	285	289	287	339	258	319	337	146	233	159	0.124
12114	107	109	114	119	118	111	102	124	128	66	84	82	0.134
13056	74	54	73	56	43	61	57	46	43	24	27	29	0.145
10946	751	628	706	717	734	712	675	734	693	570	581	470	0.155
3295	2180	2492	2271	2369	2403	2457	2278	2226	2314	3523	2228	3407	0.167
8299	4	14	17	12	9	21	9	6	11	7	14	220	0.181
15690	220	220	219	231	250	227	229	230	184	320	264	349	0.194

DE4	CC			BC			CB			BB			
#	1	2	3	1	2	3	1	2	3	1	2	3	FDR
10050	845	50	98	56	66	62	75	62	57	44	73	56	0.020
12655	31	14	24	5	4	6	2	4	8	3	6	3	0.021
11302	24	14	27	1	1	5	4	3	6	0	7	3	0.053
5079	136	6	3	4	4	5	1	7	7	3	5	6	0.073
4472	11	4	0	0	0	1	0	0	0	0	0	0	0.094
8954	444	226	336	145	154	180	185	141	199	189	160	130	0.112
8142	1350	871	870	740	776	730	763	713	792	747	702	652	0.134
12014	243	249	235	491	384	504	412	353	463	306	402	336	0.156
8532	751	750	876	972	1015	1012	1045	914	977	959	1012	1002	0.181

DE6	CC			BC			CB			BB			
#	1	2	3	1	2	3	1	2	3	1	2	3	FDR
1272	302	297	300	194	236	191	162	238	184	285	295	329	0.028
9924	614	541	411	330	358	325	342	379	376	588	367	623	0.123
8677	125	148	91	75	93	74	94	87	85	211	70	314	0.183

DE7	CC			BC			CB			BB			
#	1	2	3	1	2	3	1	2	3	1	2	3	FDR
16812	35	34	35	35	19	225	23	49	41	34	43	3423	0.015
10017	9	72	36	5	4	5	5	281	7	4	2	4	0.033
2585	1902	2186	2661	1490	1415	1432	1437	2755	1643	1488	1484	1466	0.039
9517	53	74	65	132	176	155	55	71	92	114	121	140	0.044
2853	15	90	19	6	3	3	3	209	8	1	2	4	0.074
11478	442	1536	1910	492	487	526	447	3740	583	377	376	449	0.109
16752	4	19	17	1	0	1	1	155	2	1	2	2	0.136
10383	63	88	78	25	26	23	41	173	26	26	38	21	0.165
6745	135	25	57	25	29	23	25	106	29	27	29	22	0.188

(b) Hypothalamus

DE5	CC			BC			CB			BB			
#	1	2	3	1	2	3	1	2	3	1	2	3	FDR
91	146	141	153	159	137	148	151	389	274	312	228	255	0.048
6789	33	25	44	34	36	26	31	138	127	97	80	86	0.153

Table A4. Annotated list of Ensembl identifiers (Ensembl IDs) from the RNA-seq data with FDR ≤ 0.20 using Cufflinks, Bayseq and RankProducts algorithms among the pair-wise contrast in the (a) hippocampus and (b) hypothalamus.

(a) Hippocampus

Contrast: BC *vs* CC (2nd class *vs* 1st class): up-regulated genes under 2nd class

Ensembl ID	Description
ENSGALG00000000184	solute carrier family 27 (fatty acid transporter), member 6
ENSGALG00000002744	uncharacterised
ENSGALG00000004064	G protein-coupled estrogen receptor 1
ENSGALG00000004623	angiotensin II receptor-associated protein
ENSGALG00000005209	aquaporin 1 (Colton blood group)
ENSGALG00000008139	uncharacterised
ENSGALG00000009021	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5
ENSGALG00000009308	cornichon homolog 3 (Drosophila)
ENSGALG00000010035	nuclear receptor subfamily 3, group C, member 2
ENSGALG00000011258	ATPase, Ca++ transporting, plasma membrane 1
ENSGALG00000011592	muscle RAS oncogene homolog
ENSGALG00000012440	zinc finger E-box binding homeobox 2
ENSGALG00000013925	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
ENSGALG00000014186	metallophosphoesterase domain containing 1
ENSGALG00000015271	filamin A interacting protein 1-like
ENSGALG00000016095	empty spiracles homeobox 1
ENSGALG00000016109	potassium channel, subfamily V, member 1; similar to neuronal potassium channel alpha subunit
ENSGALG00000023441	reticulon 4 receptor-like 2

Contrast: BC *vs* CC (2nd class *vs* 1st class): down-regulated genes under 2nd class

Ensembl ID	Description
ENSGALG00000000112	proteolipid protein 1 (Pelizaeus-Merzbacher disease, spastic paraplegia 2, uncomplicated)
ENSGALG00000000713	zinc finger homeobox 3
ENSGALG00000000733	myosin VIIA
ENSGALG00000000745	solute carrier family 26, member 9
ENSGALG00000001063	PR domain containing 16
ENSGALG00000001115	membrane metallo-endopeptidase-like 1
ENSGALG00000001211	hypothetical protein LOC769183
ENSGALG00000002161	similar to MGC80370 protein
ENSGALG00000002331	calbindin 2, 29kDa (calretinin)
ENSGALG00000003034	somatostatin II
ENSGALG00000003457	2',3'-cyclic nucleotide 3' phosphodiesterase
ENSGALG00000003573	hippocalcin
ENSGALG00000003770	annexin A2
ENSGALG00000004607	heme binding protein 2
ENSGALG00000004729	solute carrier family 7, (neutral amino acid transporter, y+ system) member 10
ENSGALG00000005030	dedicator of cytokinesis 10
ENSGALG00000006807	uncharacterised

ENSGALG00000006838	similar to iron binding protein
ENSGALG00000007226	osteocrin
ENSGALG00000007772	cerebellin 4 precursor
ENSGALG00000007875	endothelin converting enzyme-like 1
ENSGALG00000007945	crystallin, alpha B
ENSGALG00000008306	fibrinogen-like 2
ENSGALG00000009471	phosphatidic acid phosphatase type 2 domain containing 1A
ENSGALG00000012381	neurexophilin 2
ENSGALG00000012906	cadherin 20, type 2
ENSGALG00000013168	islet amyloid polypeptide
ENSGALG00000013362	calcium binding protein 7
ENSGALG00000013640	myelin basic protein
ENSGALG00000013890	melanocortin 5 receptor
ENSGALG00000014978	IQ motif containing GTPase activating protein 2
ENSGALG00000015143	transthyretin
ENSGALG00000015419	proenkephalin
ENSGALG00000016428	similar to autotaxin-t; ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)
ENSGALG00000016551	adaptor-related protein complex 1, sigma 2 subunit
ENSGALG00000016828	growth hormone regulated TBC protein 1
ENSGALG00000017343	folate receptor 1 (adult)
ENSGALG00000018557	similar to extracellular-superoxide dismutase (EC 1.15.1.1); superoxide dismutase 3, extracellular
ENSGALG00000021636	similar to CASP gene product
ENSGALG00000021873	hypothetical protein LOC771339
ENSGALG00000023689	argininosuccinate synthetase 1; hypothetical LOC425164

Contrast: BB *vs* CC (2nd class *vs* 1st class): up-regulated genes under 2nd class

Ensembl ID	Description
ENSGALG00000023973	alpha-1 collagen (I)

Contrast: BB *vs* CC (2nd class vs 1st class): down-regulated genes under 2nd class

Ensembl ID	Description
ENSGALG00000000713	zinc finger homeobox 3
ENSGALG00000000733	myosin VIIA
ENSGALG00000001063	PR domain containing 16
ENSGALG00000001115	membrane metallo-endopeptidase-like 1
ENSGALG00000002389	integrin, beta 4
ENSGALG00000007269	gamma-aminobutyric acid (GABA) A receptor, alpha 3
ENSGALG00000014967	synaptic vesicle glycoprotein 2C
ENSGALG00000016017	solute carrier family 4, sodium borate transporter, member 11

Contrast: CB *vs* BC (2nd class *vs* 1st class): up-regulated genes under 2nd class

Ensembl ID	Description
ENSGALG00000015143	transthyretin

Contrast: BB *vs* BC (2nd class *vs* 1st class): down-regulated genes under 2nd class

Ensembl ID	Description
ENSGALG00000006485	uncharacterised
ENSGALG00000010971	family with sequence similarity 130, member A2
ENSGALG00000013568	nuclear receptor subfamily 4, group A, member 3
ENSGALG00000017229	FAT tumor suppressor homolog 3 (Drosophila)

(b) Hypothalamus

Contrast: BB *vs* CC (2nd class *vs* 1st class): up-regulated genes under 2nd class

Ensembl ID	Description
ENSGALG00000000168	adenosine A1 receptor
ENSGALG00000000376	uncharacterised
ENSGALG00000001282	gamma-aminobutyric acid (GABA) A receptor, delta
ENSGALG00000001505	neuronal guanine nucleotide exchange factor
ENSGALG00000001727	leucine zipper, putative tumor suppressor 1
ENSGALG00000004011	uncharacterised
ENSGALG00000005721	diacylglycerol kinase, gamma 90kDa
ENSGALG00000005853	5-hydroxytryptamine (serotonin) receptor 2C
ENSGALG00000006021	calcium channel, voltage-dependent, gamma subunit 3
ENSGALG00000006406	bombesin-like receptor 3
ENSGALG00000006576	glutamate receptor, metabotropic 3
ENSGALG00000007004	5-hydroxytryptamine (serotonin) receptor 3A
ENSGALG00000007141	leucine-rich repeat kinase 1
ENSGALG00000008135	SATB homeobox 2
ENSGALG00000008308	basic helix-loop-helix domain containing, class B, 2
ENSGALG00000008365	cholinergic receptor, muscarinic 4
ENSGALG00000008885	phosphodiesterase 1A, calmodulin-dependent
ENSGALG00000008940	spectrin, beta, non-erythrocytic 5
ENSGALG00000009853	forkhead box G1
ENSGALG00000009859	TBC1 domain family, member 30
ENSGALG00000010939	lin-7 homolog A (*C. elegans*)
ENSGALG00000011122	uncharacterised

ENSGALG00000011254	SATB homeobox 1
ENSGALG00000011613	copine IV
ENSGALG00000011721	A kinase (PRKA) anchor protein 5
ENSGALG00000011883	C-type lectin domain family 3, member B
ENSGALG00000012235	neurogenic differentiation 6
ENSGALG00000012367	tripartite motif-containing 9
ENSGALG00000012542	RASD family, member 2
ENSGALG00000012732	phosphatase and actin regulator 1
ENSGALG00000012890	diacylglycerol kinase, iota
ENSGALG00000013795	human immunodeficiency virus type I enhancer binding protein 2
ENSGALG00000013948	RAS-like, family 11, member B
ENSGALG00000014634	silver homolog (mouse)
ENSGALG00000014645	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)
ENSGALG00000014907	discoidin, CUB and LCCL domain containing 1
ENSGALG00000015271	filamin A interacting protein 1-like
ENSGALG00000015626	regulator of G-protein signalling 12
ENSGALG00000015842	T-cell lymphoma invasion and metastasis 1
ENSGALG00000016154	activity-regulated cytoskeleton-associated protein
ENSGALG00000016920	LIM domain 7
ENSGALG00000018942	neurogranin (protein kinase C substrate, RC3)
ENSGALG00000019842	transcription factor AP-2delta

Contrast: BB *vs* BC (2nd class *vs* 1st class): up-regulated genes under 2nd class

Ensembl ID	Description
ENSGALG00000000168	adenosine A1 receptor
ENSGALG00000000695	major facilitator superfamily domain containing 4
ENSGALG00000001564	ATPase, Ca++ transporting, ubiquitous
ENSGALG00000007004	5-hydroxytryptamine (serotonin) receptor 3A
ENSGALG00000007278	glutamate receptor, ionotropic, N-methyl D-aspartate 2A

ENSGALG00000008135	SATB homeobox 2
ENSGALG00000008940	spectrin, beta, non-erythrocytic 5
ENSGALG00000009556	prickle homolog 1 (Drosophila)
ENSGALG00000011122	uncharacterised
ENSGALG00000012183	neuronal pentraxin receptor
ENSGALG00000012235	neurogenic differentiation 6
ENSGALG00000012254	potassium inwardly-rectifying channel, subfamily J, member 4
ENSGALG00000018942	neurogranin (protein kinase C substrate, RC3)
ENSGALG00000019842	transcription factor AP-2delta
ENSGALG00000022001	hypothetical LOC415928
ENSGALG00000023441	reticulon 4 receptor-like 2

Table A5. Top 20 up- or down-regulated significant (FDR ≤ 0.10) transcripts across the pair-wise contrasts in the (a) hippocampus and (b) hypothalamus. Genes were sorted according to the RankProducts statistics in ascending order and cut at the level of FDR 0.1. FC denotes the fold change.

(a) Hippocampus

Contrast: BC *vs* CC

(1) Top 20 up-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000006137	Rho GTPase activating protein 22	0.0020	1.9148
ENSGALG00000012544	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-cetylgalactosaminyltransferase 5 (GalNAc-T5)	0.0023	1.9347
ENSGALG00000008723	complement component 1, q subcomponent-like 3	0.0025	1.9635
ENSGALG00000016465	similar to egg envelope component ZPAX	0.0040	1.9502
ENSGALG00000014414	gamma-aminobutyric acid (GABA) receptor, rho 3	0.0050	1.9303
ENSGALG00000012327	inhibin, beta A	0.0051	1.8766
ENSGALG00000000184	solute carrier family 27 (fatty acid transporter), member 6	0.0053	1.8676
ENSGALG00000008135	SATB homeobox 2	0.0053	1.7825
ENSGALG00000006676	retinaldehyde binding protein 1	0.0060	1.9513
ENSGALG00000016095	empty spiracles homeobox 1	0.0064	1.7828
ENSGALG00000016499	hypothetical protein LOC770429	0.0075	1.7465
ENSGALG00000000507	copine VII	0.0078	1.7435
ENSGALG00000005347	similar to ADAMTS18 protein	0.0079	1.7224
ENSGALG00000005802	fms-related tyrosine kinase 4	0.0082	1.7570
ENSGALG00000016500	FK506 binding protein 1B, 12.6 kDa	0.0083	1.7559
ENSGALG00000005772	BCL2-related ovarian killer	0.0085	1.7838
ENSGALG00000015720	chondrolectin	0.0088	1.7736
ENSGALG00000015271	filamin A interacting protein 1-like	0.0090	1.6675
ENSGALG00000013925	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	0.0096	1.6287
ENSGALG00000017064	replication factor C (activator 1) 3, 38kDa	0.0114	1.6222

(2) Top 20 down-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000001696	S-antigen; retina and pineal gland (arrestin)	< 0.0001	-18.196
ENSGALG00000013154	solute carrier organic anion transporter family, member 1C1	< 0.0001	-8.0053
ENSGALG00000015143	transthyretin	< 0.0001	-5.8174
ENSGALG00000007875	endothelin converting enzyme-like 1	0.0003	-3.2126
ENSGALG00000011859	eye-globin	0.0012	-3.5947
ENSGALG00000011369	LIM homeobox 8	0.0013	-3.6024
ENSGALG00000016553	transmembrane protein 27	0.0015	-3.2546
ENSGALG00000001490	uncharacterised	0.0017	-2.8839

ENSGALG00000018557	similar to extracellular-superoxide dismutase (EC 1.15.1.1); superoxide dismutase 3, extracellular	0.0018	-2.8748
ENSGALG00000012908	solute carrier family 13 (sodium/sulfate symporters), member 4	0.0019	-3.6478
ENSGALG00000007367	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2	0.0019	-2.6331
ENSGALG00000016020	chloride intracellular channel 6	0.0021	-2.9338
ENSGALG00000017343	folate receptor 1 (adult)	0.0027	-2.4622
ENSGALG00000007179	ATPase type 13A5	0.0028	-2.6189
ENSGALG00000003034	somatostatin II	0.0028	-2.4014
ENSGALG00000015918	EF-hand calcium binding protein 1	0.0030	-2.3182
ENSGALG00000015595	G protein-coupled receptor 78	0.0031	-2.3334
ENSGALG00000000733	myosin VIIA	0.0031	-2.3271
ENSGALG00000003573	hippocalcin	0.0033	-2.4069
ENSGALG00000013168	islet amyloid polypeptide	0.0041	-2.2804

Contrast: CB *vs* CC

(1) Top 20 up-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000002577	StAR-related lipid transfer (START) domain containing 10	0.0300	1.9365
ENSGALG00000004322	uncharacterised	0.0773	1.6685
ENSGALG00000014414	gamma-aminobutyric acid (GABA) receptor, rho 3	0.0840	1.8285

(2) Top 20 down-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000013154	solute carrier organic anion transporter family, member 1C1	0.0045	-3.3707
ENSGALG00000012908	solute carrier family 13 (sodium/sulfate symporters), member 4	0.0050	-2.8656
ENSGALG00000007367	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2	0.0090	-2.6494
ENSGALG00000011859	eye-globin	0.0205	-2.6732
ENSGALG00000015143	transthyretin	0.0242	-4.1646
ENSGALG00000001490	uncharacterised	0.0427	-2.1829
ENSGALG00000016553	transmembrane protein 27	0.0441	-2.2201
ENSGALG00000001696	S-antigen; retina and pineal gland (arrestin)	0.0503	-2.9875
ENSGALG00000011858	potassium voltage-gated channel, subfamily H (eag-related), member 5	0.0530	-1.9626
ENSGALG00000010934	InaD-like (Drosophila)	0.0650	-1.9627

ENSGALG00000018557	similar to extracellular-superoxide dismutase (EC 1.15.1.1); superoxide dismutase 3, extracellular	0.0673	-2.0535
ENSGALG00000017343	folate receptor 1 (adult)	0.0718	-1.9214
ENSGALG00000016017	solute carrier family 4, sodium borate transporter, member 11	0.0871	-1.8846
ENSGALG00000007179	ATPase type 13A5	0.0915	-1.9503
ENSGALG00000013515	solute carrier family 4, sodium bicarbonate cotransporter, member 5; similar to sodium bicarbonate	0.0940	-1.8976
ENSGALG00000008874	solute carrier family 13 (sodium/sulfate symporters), member 1	0.0943	-1.9148
ENSGALG00000016554	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	0.0952	-1.8784
ENSGALG00000006838	similar to iron binding protein	0.0970	-1.6450
ENSGALG00000011813	HEG homolog 1 (zebrafish)	0.0974	-1.7349
ENSGALG00000017059	mab-21-like 1 (*C. elegans*)	0.0979	-2.0561

Contrast: BB *vs* CC

(1) Top 20 up-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000012226	chromosome 7 open reading frame 16	0.0473	3.1520
ENSGALG00000004322	uncharacterised	0.0502	1.9054
ENSGALG00000015018	calsequestrin 2 (cardiac muscle)	0.0533	3.1243
ENSGALG00000015857	carbonic anhydrase III, muscle specific	0.0548	1.9855
ENSGALG00000008193	reelin	0.0615	2.4139
ENSGALG00000012285	BAI1-associated protein 2-like 2	0.0620	3.2025
ENSGALG00000005985	growth differentiation factor 10	0.0683	2.1699
ENSGALG00000024278	uncharacterised	0.0725	2.8673
ENSGALG00000003894	cerebellin 1 precursor	0.0730	2.9982
ENSGALG00000008908	neurogenic differentiation 1	0.0756	2.1326
ENSGALG00000004527	hypothetical protein LOC776119	0.0852	2.0334
ENSGALG00000023430	uncharacterised	0.0865	2.4479
ENSGALG00000001695	gamma-aminobutyric acid (GABA) A receptor, alpha 6	0.0878	2.6068
ENSGALG00000000920	cingulin	0.0896	2.5918
ENSGALG00000012544	UDP-N-acetyl-alpha-D-galactosamine:	0.0962	1.6314
ENSGALG00000009241	secreted frizzled-related protein 2	0.0968	2.0671
ENSGALG00000017417	similar to ubiquitin specific proteinase 43	0.0984	2.2327
ENSGALG00000000441	potassium voltage-gated channel,	0.0994	2.5667
ENSGALG00000005842	glycerophosphodiester phosphodiesteras e	0.0995	2.8442

(2) Top 20 down-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000015143	transthyretin	< 0.0001	-28.0977
ENSGALG00000013154	solute carrier organic anion transporter	< 0.0001	-6.5400
ENSGALG00000011859	eye-globin	< 0.0001	-4.5587
ENSGALG00000001696	S-antigen; retina and pineal gland (arrestin)	< 0.0001	-9.8715
ENSGALG00000007367	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2	0.0003	-3.0221
ENSGALG00000016553	transmembrane protein 27	0.0003	-3.5302
ENSGALG00000001490	uncharacterised	0.0004	-3.2100
ENSGALG00000016020	chloride intracellular channel 6	0.0004	-3.3355
ENSGALG00000000733	myosin VIIA	0.0004	-2.8969
ENSGALG00000016017	solute carrier family 4, sodium borate	0.0007	-2.7615
ENSGALG00000012908	solute carrier family 13 (sodium/sulfate	0.0014	-3.7024
ENSGALG00000014967	synaptic vesicle glycoprotein 2C	0.0018	-2.4284
ENSGALG00000001063	PR domain containing 16	0.0030	-2.2823
ENSGALG00000017343	folate receptor 1 (adult)	0.0031	-2.3754
ENSGALG00000001115	membrane metallo-endopeptidase-like 1	0.0032	-2.4286
ENSGALG00000018557	similar to extracellular-superoxide dismutase	0.0032	-2.8380
ENSGALG00000011369	LIM homeobox 8	0.0033	-3.2484
ENSGALG00000015419	proenkephalin	0.0043	-2.6196
ENSGALG00000005628	collagen, type IX, alpha 3	0.0065	-2.7236
ENSGALG00000017068	klotho	0.0072	-2.2514

Contrast: CB *vs* BC

(1) Top 20 up-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000015143	transthyretin	0.0010	4.3747
ENSGALG00000014117	arginine vasopressin (neurophysin II, antidiuretic hormone, diabetes insipidus, neurohypophyseal)	0.0020	4.2093
ENSGALG00000001696	S-antigen; retina and pineal gland (arrestin)	0.0373	2.7064
ENSGALG00000020975	uncharacterised	0.0465	1.7710

(2) Top 20 down-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000004320	FAT tumor suppressor homolog 2 (Drosophila)	0.0020	-2.6134
ENSGALG00000008723	complement component 1, q subcomponent-like 3	0.0080	-1.8987

ENSGALG00000005842	glycerophosphodiester phosphodiesterase domain containing 2	0.0115	-2.4758
ENSGALG00000012544	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-etylgalactosaminyltransferase 5 (GalNAc-T5)	0.0128	-1.7961
ENSGALG00000012285	BAI1-associated protein 2-like 2	0.0320	-1.8686
ENSGALG00000008135	SATB homeobox 2	0.0338	-1.5855
ENSGALG00000009431	uncharacterised	0.0881	-1.6929
ENSGALG00000005802	fms-related tyrosine kinase 4	0.0906	-1.3155
ENSGALG00000016465	similar to egg envelope component ZPAX	0.0907	-1.5696
ENSGALG00000002945	chromosome 15 open reading frame 27	0.0948	-1.5645

Contrast: BB *vs* BC

(1) Top 20 up-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000000681	similar to PAK3 protein; p21 (CDKN1A)-activated kinase 3; p21 protein	0.0522	1.9490
ENSGALG00000000920	cingulin	0.0373	2.9528
ENSGALG00000001172	kainate binding protein	0.0506	2.6320
ENSGALG00000002161	similar to MGC80370 protein	0.0495	2.0372
ENSGALG00000003354	potassium voltage-gated channel, subfamily H (eag-related), member 4	0.0471	1.9794
ENSGALG00000003894	cerebellin 1 precursor	0.0350	2.9906
ENSGALG00000004320	FAT tumor suppressor homolog 2 (Drosophila)	0.0284	2.6243
ENSGALG00000004527	hypothetical protein LOC776119; unc-13 homolog C (*C. elegans*); similar to	0.0364	3.0010
ENSGALG00000005409	LIM homeobox 1	0.0526	2.6154
ENSGALG00000005842	glycerophosphodiester phosphodiesterase domain containing 2	0.0486	2.3907
ENSGALG00000006811	Zic family member 1 (odd-paired homolog, Drosophila)	0.0404	2.9334
ENSGALG00000008193	reelin	0.0379	2.6058
ENSGALG00000008945	nexilin (F actin binding protein)	0.0228	2.8774
ENSGALG00000009012	zinc finger protein 533	0.0242	3.2279
ENSGALG00000009431	uncharacterised	0.0490	2.5611
ENSGALG00000010934	InaD-like (Drosophila)	0.0499	2.5613
ENSGALG00000011262	potassium voltage-gated channel, subfamily H (eag-related), member 8	0.0293	2.2552
ENSGALG00000012226	chromosome 7 open reading frame 16	0.0409	2.1956

ENSGALG00000015018	calsequestrin 2 (cardiac muscle)	0.0485	2.4365
ENSGALG00000023430	uncharacterised	0.0394	3.0591

(2) Top 20 down-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000006473	plexin A4, B	0.0160	-1.8857
ENSGALG00000017173	guanylate cyclase 1, soluble, alpha 2	0.0292	-1.7813
ENSGALG00000005347	similar to ADAMTS18 protein	0.0307	-1.9930
ENSGALG00000007871	similar to similar to glutamate transporter 1 variant;	0.0310	-1.8935
ENSGALG00000014967	synaptic vesicle glycoprotein 2C	0.0310	-1.8210
ENSGALG00000011577	contactin associated protein-like 5	0.0329	-1.7609
ENSGALG00000008885	phosphodiesterase 1A, calmodulin-dependent	0.0362	-1.7029
ENSGALG00000006485	uncharacterised	0.0378	-1.7609
ENSGALG00000005802	fms-related tyrosine kinase 4	0.0405	-1.6421
ENSGALG00000001608	unc-5 homolog D (*C. elegans*)	0.0483	-1.6998
ENSGALG00000015080	solute carrier family 24 (sodium/potassium/calcium exchanger),	0.0513	-1.6969
ENSGALG00000009737	tachykinin, precursor 1 (substance K, substance P, neurokinin 1, neurokinin 2,	0.0525	-1.6675
ENSGALG00000017281	uncharacterised	0.0525	-1.6908
ENSGALG00000012248	similar to MAP3K9 protein; mitogen-activated protein kinase kinase kinase 9	0.0528	-1.6407
ENSGALG00000008723	complement component 1, q subcomponent-like 3	0.0541	-1.6211
ENSGALG00000002799	chromosome 2 open reading frame 21	0.0554	-1.6813
ENSGALG00000008544	similar to Na+/Ca2+ exchanger; solute carrier family 8	0.0567	-1.6168
ENSGALG00000000184	solute carrier family 27 (fatty acid transporter), member 6	0.0568	-1.6835
ENSGALG00000015737	neural cell adhesion molecule 2	0.0569	-1.6369
ENSGALG00000007993	doublecortex	0.0569	-1.6449

Contrast: BB *vs* CB

(1) Top 20 up-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000003894	cerebellin 1 precursor	0.0095	4.4751
ENSGALG00000004320	FAT tumor suppressor homolog 2 (Drosophila)	0.0100	6.8750
ENSGALG00000005842	glycerophosphodiester phosphodiesterase domain containing 2	0.0108	5.9259
ENSGALG00000012285	BAI1-associated protein 2-like 2	0.0117	6.1420
ENSGALG00000000920	cingulin	0.0122	4.6988
ENSGALG00000004527	hypothetical protein LOC776119; unc-13 homolog C (C. elegans); similar to Munc13-3	0.0133	4.4623
ENSGALG00000024278	uncharacterised	0.0164	3.5586
ENSGALG00000006811	Zic family member 1 (odd-paired homolog, Drosophila)	0.0181	3.4901
ENSGALG00000008945	nexilin (F actin binding protein)	0.0220	2.8214
ENSGALG00000009012	zinc finger protein 533	0.0238	3.4529
ENSGALG00000009431	uncharacterised	0.0245	4.3605
ENSGALG00000012226	chromosome 7 open reading frame 16	0.0254	3.1649
ENSGALG00000008908	neurogenic differentiation 1	0.0258	2.5441
ENSGALG00000003354	potassium voltage-gated channel, subfamily H (eag-related), member 4	0.0268	2.1464
ENSGALG00000016988	chromosome 13 open reading frame 18	0.0328	3.3360
ENSGALG00000008193	reelin	0.0331	3.6453
ENSGALG00000023430	uncharacterised	0.0333	3.8019
ENSGALG00000015018	calsequestrin 2 (cardiac muscle)	0.0335	3.2215
ENSGALG00000003149	inositol 1,4,5-triphosphate receptor, type 3	0.0336	2.9759
ENSGALG00000015778	gamma-aminobutyric acid (GABA) receptor, rho 1	0.0337	3.5362

(2) Top 20 down-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000015143	transthyretin	0.0020	-6.7550
ENSGALG00000001696	S-antigen; retina and pineal gland (arrestin)	0.0590	-3.3422

(b) Hypothalamus

Contrast: BC *vs* CC

(1) Top 20 up-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000009740	PR domain containing 16	0.0000	3.4815
ENSGALG00000011369	LIM homeobox 8	0.0005	2.3797
ENSGALG00000015529	Wolfram syndrome 1 (wolframin)	0.0007	2.4260
ENSGALG00000017194	transient receptor potential cation channel, subfamily C, member 6	0.0016	2.0623

ENSGALG00000007980	phosphodiesterase 1A, calmodulin-dependent	0.0016	2.2307
ENSGALG00000001074	LIM homeobox 6	0.0018	2.1270
ENSGALG00000001347	PR domain containing 12	0.0020	2.0098
ENSGALG00000017044	transient receptor potential cation channel, subfamily C, member 4	0.0041	2.1187
ENSGALG00000001063	hypothetical gene supported by CR385622	0.0041	1.9595
ENSGALG00000007139	potassium voltage-gated channel, subfamily G, member 1	0.0051	2.0885
ENSGALG00000009853	forkhead box G1	0.0055	1.9958
ENSGALG00000014804	thrombospondin 4	0.0066	1.8216
ENSGALG00000003895	family with sequence similarity 107, member A	0.0069	2.0431
ENSGALG00000015857	carbonic anhydrase III, muscle specific	0.0072	1.8534
ENSGALG00000014843	tumor protein D52-like 1	0.0076	1.9745
ENSGALG00000008885	similar to RAS guanyl releasing protein 1 (calcium and DAG-regulated); RAS guanyl releasing protein 1 (calcium and DAG-regulated)	0.0084	1.8739
ENSGALG00000009799	Meis homeobox 2	0.0085	1.8950
ENSGALG00000011170	WAS/WASL interacting protein family, member 3	0.0132	1.8970
ENSGALG00000014484	uncharacterised	0.0207	1.8127
ENSGALG00000016866	fibroblast growth factor 14	0.0358	1.6506

(2) Top 20 down-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000000507	copine VII	0.0080	-2.7013
ENSGALG00000003839	glutamate receptor, metabotropic 2	0.0110	-2.4097
ENSGALG00000009095	luteinizing hormone/choriogonadotropin receptor	0.0118	-2.2222
ENSGALG00000008883	transcription factor 7-like 2 (T-cell specific, HMG-box)	0.0120	-2.2245
ENSGALG00000004919	uncharacterised	0.0135	-3.0885
ENSGALG00000009791	prospero-related homeobox 1	0.0185	-1.9404
ENSGALG00000002331	calbindin 2, 29kDa (calretinin)	0.0207	-1.8891
ENSGALG00000024278	uncharacterised	0.0207	-1.6992
ENSGALG00000006838	similar to iron binding protein	0.0211	-1.6637
ENSGALG00000003894	cerebellin 1 precursor	0.0213	-1.7183
ENSGALG00000003562	neuronal pentraxin II	0.0231	-1.8905

ENSGALG00000016600	proopiomelanocortin (adrenocorticotropin/ beta-lipotropin/ alpha-melanocyte stimulating hormone/ beta-melanocyte stimulating hormone/ beta-endorphin)	0.0234	-1.5726
ENSGALG00000014477	CD4 molecule	0.0234	-2.0514
ENSGALG00000013193	iroquois homeobox 2	0.0304	-1.9073
ENSGALG00000002223	LIM homeobox 9	0.0343	-1.8817
ENSGALG00000016083	similar to Angiopoietin 1; angiopoietin 1	0.0350	-1.8203
ENSGALG00000021567	uncharacterised	0.0353	-1.7714
ENSGALG00000008735	beaded filament structural protein 1, filensin	0.0377	-1.8273
ENSGALG00000015824	glycoprotein hormones, alpha polypeptide	0.0536	-1.6450
ENSGALG00000016904	SLIT and NTRK-like family, member 6	0.0555	-1.7264

Contrast: CB *vs* CC

(1) Top 20 up-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000008135	SATB homeobox 2	0.0037	3.0762
ENSGALG00000014907	discoidin, CUB and LCCL domain containing 1	0.0038	3.2824
ENSGALG00000002821	gastrin-releasing peptide	0.0040	3.0643
ENSGALG00000012235	neurogenic differentiation 6	0.0055	2.9570
ENSGALG00000006406	bombesin-like receptor 3	0.0122	2.8584
ENSGALG00000014011	lymphoid-restricted membrane protein	0.0168	2.4149
ENSGALG00000001282	gamma-aminobutyric acid (GABA) A receptor, delta	0.0170	2.3082
ENSGALG00000015626	regulator of G-protein signalling 12	0.0171	2.5448
ENSGALG00000004270	aldehyde dehydrogenase 1 family, member A2	0.0175	2.5447
ENSGALG00000007141	leucine-rich repeat kinase 1	0.0184	2.6677
ENSGALG00000011122	uncharacterised	0.0208	2.7867
ENSGALG00000015271	filamin A interacting protein 1-like	0.0247	2.3942
ENSGALG00000014645	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)	0.0321	2.2620
ENSGALG00000008885	phosphodiesterase 1A, calmodulin-dependent	0.0367	2.2236
ENSGALG00000016920	LIM domain 7	0.0371	2.1656
ENSGALG00000019842	transcription factor AP-2delta	0.0374	2.2918
ENSGALG00000023441	reticulon 4 receptor-like 2	0.0406	1.9892
ENSGALG00000018942	neurogranin (protein kinase C substrate, RC3)	0.0765	1.9119
ENSGALG00000020515	uncharacterised	0.0794	1.9019
ENSGALG00000012254	potassium inwardly-rectifying channel, subfamily J, member 4	0.0853	2.0097

(2) Top 20 down-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000004919	uncharacterised	0.0020	-4.6928
ENSGALG00000015143	transthyretin	0.0065	-4.1969
ENSGALG00000004572	natriuretic peptide precursor C	0.0467	-2.2076
ENSGALG00000003894	cerebellin 1 precursor	0.0510	-2.0654
ENSGALG00000012464	SOUL protein	0.0740	-2.1329

Contrast: BB *vs* CC

(1) Top 20 up-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000006406	bombesin-like receptor 3	0.0000	3.3041
ENSGALG00000008135	SATB homeobox 2	0.0000	4.4087
ENSGALG00000011122	uncharacterised	0.0000	3.4907
ENSGALG00000012235	neurogenic differentiation 6	0.0000	4.0161
ENSGALG00000014907	discoidin, CUB and LCCL domain containing 1	0.0000	4.2754
ENSGALG00000014645	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)	0.0001	2.5816
ENSGALG00000001282	gamma-aminobutyric acid (GABA) A receptor, delta	0.0001	2.7574
ENSGALG00000004270	aldehyde dehydrogenase 1 family, member A2	0.0001	2.9466
ENSGALG00000015626	regulator of G-protein signalling 12	0.0001	2.7538
ENSGALG00000007141	leucine-rich repeat kinase 1	0.0001	2.8333
ENSGALG00000008885	phosphodiesterase 1A, calmodulin-dependent	0.0002	2.5087
ENSGALG00000009740	similar to RAS guanyl releasing protein 1 (calcium and DAG-regulated); RAS guanyl releasing protein 1 (calcium and DAG-regulated)	0.0002	2.5902
ENSGALG00000019842	transcription factor AP-2delta	0.0002	2.9304
ENSGALG00000014011	lymphoid-restricted membrane protein	0.0004	2.5451
ENSGALG00000015271	filamin A interacting protein 1-like	0.0006	2.3232
ENSGALG00000009853	forkhead box G1	0.0006	2.3101
ENSGALG00000011721	A kinase (PRKA) anchor protein 5	0.0008	2.2408
ENSGALG00000018942	neurogranin (protein kinase C substrate, RC3)	0.0008	2.2257
ENSGALG00000013154	solute carrier organic anion transporter family, member 1C1	0.0010	2.3958
ENSGALG00000011254	SATB homeobox 1	0.0013	2.1602

(2) Top 20 down-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000004919	uncharacterised	0.0000	-4.8313
ENSGALG00000009791	prospero-related homeobox 1	0.0425	-1.8623
ENSGALG00000014477	CD4 molecule	0.0427	-1.8734
ENSGALG00000008900	tetra-peptide repeat homeobox-like	0.0544	-1.5701

ENSGALG00000009095	luteinizing hormone/choriogonadotropin receptor	0.0555	-1.7583
ENSGALG00000002331	calbindin 2, 29kDa (calretinin)	0.0565	-1.6316
ENSGALG00000016904	SLIT and NTRK-like family, member 6	0.0571	-1.7489
ENSGALG00000002223	LIM homeobox 9	0.0597	-1.7723
ENSGALG00000008883	transcription factor 7-like 2 (T-cell specific, HMG-box)	0.0624	-1.7920
ENSGALG00000006236	tryptophan hydroxylase 1	0.0693	-1.4720
ENSGALG00000013193	iroquois homeobox 2	0.0694	-1.7682
ENSGALG00000012911	synaptotagmin X	0.0700	-1.7574
ENSGALG00000012495	uncharacterised	0.0822	-1.6020
ENSGALG00000007772	cerebellin 4 precursor	0.0841	-1.6096
ENSGALG00000008671	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6	0.0859	-1.5017
ENSGALG00000010461	early B-cell factor 3	0.0869	-1.5718
ENSGALG00000010402	prostaglandin-D synthase	0.0909	-1.4618
ENSGALG00000023036	uncharacterised	0.0931	-1.5130
ENSGALG00000006384	interferon-induced protein with tetratricopeptide repeats 5	0.0956	-1.6101
ENSGALG00000003894	cerebellin 1 precursor	0.0983	-1.4688

Contrast: CB *vs* BC

(1) Top 20 up-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000002821	gastrin-releasing peptide	0.0010	2.8454
ENSGALG00000012235	neurogenic differentiation 6	0.0020	3.7793
ENSGALG00000008135	SATB homeobox 2	0.0083	2.8284
ENSGALG00000007141	leucine-rich repeat kinase 1	0.0172	2.4867
ENSGALG00000004270	aldehyde dehydrogenase 1 family, member A2	0.0200	2.4217
ENSGALG00000014011	lymphoid-restricted membrane protein	0.0200	2.4001
ENSGALG00000023441	reticulon 4 receptor-like 2	0.0204	2.2214
ENSGALG00000001564	ATPase, Ca++ transporting, ubiquitous	0.0205	2.7068
ENSGALG00000019842	transcription factor AP-2delta	0.0206	2.4529
ENSGALG00000014907	discoidin, CUB and LCCL domain containing 1	0.0298	2.1306
ENSGALG00000011271	lumican	0.0318	1.8437
ENSGALG00000011122	uncharacterised	0.0322	1.8973
ENSGALG00000000507	copine VII	0.0328	2.1166
ENSGALG00000012254	potassium inwardly-rectifying channel, subfamily J, member 4	0.0328	2.2816
ENSGALG00000015271	filamin A interacting protein 1-like	0.0342	2.2372
ENSGALG00000018942	neurogranin (protein kinase C substrate, RC3)	0.0346	1.8758
ENSGALG00000003839	glutamate receptor, metabotropic 2	0.0352	2.0836

ENSGALG00000008908	neurogenic differentiation 1	0.0361	2.1278
ENSGALG00000008032	G protein-coupled receptor 22	0.0374	2.1532
ENSGALG00000015626	regulator of G-protein signalling 12	0.0424	1.9791

(2) Top 20 down-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000001063	PR domain containing 16	0.0230	-2.0024
ENSGALG00000003149	inositol 1,4,5-triphosphate receptor, type 3	0.0660	-2.0682
ENSGALG00000003895	PR domain containing 12	0.0546	-1.8305
ENSGALG00000006112	sodium channel, voltage-gated, type V, alpha subunit	0.0327	-1.9275
ENSGALG00000007047	galanin prepropeptide	0.0549	-1.9036
ENSGALG00000007972	transient receptor potential cation channel, subfamily C, member 5	0.0556	-1.7112
ENSGALG00000008621	similar to neuropilin-2a1 receptor; neuropilin 2	0.0514	-1.6908
ENSGALG00000009173	GDNF family receptor alpha 1	0.0630	-1.6382
ENSGALG00000009740	similar to RAS guanyl releasing protein 1 (calcium and DAG-regulated); RAS guanyl releasing protein 1 (calcium and DAG-regulated)	0.0358	-1.8443
ENSGALG00000011022	neuropeptide VF precursor	0.0625	-1.5799
ENSGALG00000011369	LIM homeobox 8	0.0600	-1.7260
ENSGALG00000012464	SOUL protein	0.0445	-2.1554
ENSGALG00000013294	cytochrome P450, family 19, subfamily A, polypeptide 1	0.0459	-2.0576
ENSGALG00000014484	uncharacterised	0.0584	-1.7314
ENSGALG00000014843	tumor protein D52-like 1	0.0541	-1.8108
ENSGALG00000015143	transthyretin	0.0635	-2.5067
ENSGALG00000016455	uncharacterised	0.0560	-1.8658
ENSGALG00000016707	chloride intracellular channel 5	0.0554	-1.7380
ENSGALG00000017044	transient receptor potential cation channel, subfamily C, member 4	0.0258	-2.0181
ENSGALG00000017194	transient receptor potential cation channel, subfamily C, member 6	0.0522	-1.7468

Contrast: BB *vs* BC

(1) Top 20 up-regulated genes under 2nd class

		FDR	FC
ENSGALG00000001564	ATPase, Ca++ transporting, ubiquitous	<0.0001	2.8468
ENSGALG00000004270	aldehyde dehydrogenase 1 family, member A2	<0.0001	2.8057
ENSGALG00000008135	SATB homeobox 2	<0.0001	4.0256
ENSGALG00000012235	neurogenic differentiation 6	<0.0001	5.1060
ENSGALG00000019842	transcription factor AP-2delta	<0.0001	3.0998
ENSGALG00000011122	Uncharacterised	0.00013	2.3724
ENSGALG00000013154	solute carrier organic anion transporter family, member 1C1	0.00014	2.6388
ENSGALG00000014907	discoidin, CUB and LCCL domain containing 1	0.00017	2.7727
ENSGALG00000023441	reticulon 4 receptor-like 2	0.00018	2.2530
ENSGALG00000012254	potassium inwardly-rectifying channel, subfamily J, member 4	2.00E-04	2.2675
ENSGALG00000007141	leucine-rich repeat kinase 1	0.00022	2.6386
ENSGALG00000003839	glutamate receptor, metabotropic 2	0.00031	2.3058
ENSGALG00000018942	neurogranin (protein kinase C substrate, RC3)	0.00033	2.1826
ENSGALG00000015271	filamin A interacting protein 1-like	0.00071	2.1682
ENSGALG00000014829	R-spondin 3 homolog (*Xenopus laevis*)	0.00138	2.0225
ENSGALG00000008032	G protein-coupled receptor 22	0.0014	2.1012
ENSGALG00000014011	lymphoid-restricted membrane protein	0.00141	2.4858
ENSGALG00000015626	regulator of G-protein signalling 12	0.00189	2.1415
ENSGALG00000008940	spectrin, beta, non-erythrocytic 5	0.00205	1.8481
ENSGALG00000000820	5-hydroxytryptamine (serotonin) receptor 1D	0.0043	1.8261

(2) Top 20 down-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000013294	cytochrome P450, family 19, subfamily A, polypeptide 1	0.0060	-1.7109
ENSGALG00000004754	obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	0.0070	-1.7063
ENSGALG00000011369	LIM homeobox 8	0.0080	-1.7391
ENSGALG00000011973	sushi domain containing 5	0.0150	-1.7180
ENSGALG00000014967	synaptic vesicle glycoprotein 2C	0.0168	-1.5968
ENSGALG00000019144	gamma-aminobutyric acid (GABA) A receptor, gamma 3	0.0275	-1.5698
ENSGALG00000006112	sodium channel, voltage-gated, type V, alpha subunit	0.0389	-1.5659
ENSGALG00000006473	plexin A4, B	0.0717	-1.4899

ENSGALG00000012495	uncharacterised	0.0722	-1.4865
ENSGALG00000009173	GDNF family receptor alpha 1	0.0724	-1.5144
ENSGALG00000007972	transient receptor potential cation channel, subfamily C, member 5	0.0775	-1.5231
ENSGALG00000015529	Wolfram syndrome 1 (wolframin)	0.0778	-1.4117
ENSGALG00000014717	uncharacterised	0.0789	-1.4625
ENSGALG00000004838	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	0.0799	-1.5141
ENSGALG00000006485	uncharacterised	0.0836	-1.4800
ENSGALG00000012324	chromosome 7 open reading frame 10	0.0859	-1.4490
ENSGALG00000013177	branched chain aminotransferase 1, cytosolic	0.0872	-1.4502
ENSGALG00000001608	unc-5 homolog D (*C. elegans*)	0.0952	-1.4394
ENSGALG00000016804	solute carrier family 5 (choline transporter), member 7	0.0979	-1.4437

Contrast: BB *vs* CB

(1) Top 20 up-regulated genes under 2nd class

Ensembl ID	Description	FDR	FC
ENSGALG00000015143	transthyretin	0.0020	7.8399
ENSGALG00000001696	S-antigen; retina and pineal gland (arrestin)	0.0200	3.2449
ENSGALG00000014634	silver homolog (mouse)	0.0300	2.3372
ENSGALG00000016020	chloride intracellular channel 6	0.0610	1.9277
ENSGALG00000001063	PR domain containing 16	0.0686	1.7119
ENSGALG00000007179	ATPase type 13A5	0.0688	1.9963
ENSGALG00000011859	eye-globin	0.0710	1.9526
ENSGALG00000008941	uncharacterised	0.0711	1.6936
ENSGALG00000013154	solute carrier organic anion transporter family, member 1C1	0.0855	2.4170
ENSGALG00000009867	WNT inhibitory factor 1	0.0947	1.7129

(2) Top 20 down-regulated genes under 2nd class

ENSGALG00000014118	mitochondrial ribosomal protein S26	0.0000	-2.2201
ENSGALG00000018808	mitochondrial ribosomal protein S26	0.0075	-1.9027
ENSGALG00000002821	gastrin-releasing peptide	0.0423	-1.7801

Table A6. List of genes that met the behavioural filtering categories in the (a) hippocampus and (b) hypothalamus as described in detail in the Methods (Chapter 4, Section 4.3.13.1).

(a) Hippocampus

Pre- and post-hatching B responsive genes

Ensembl ID	Description
ENSGALG00000000713	zinc finger homeobox 3
ENSGALG00000000745	solute carrier family 26, member 9
ENSGALG00000001115	membrane metallo-endopeptidase-like 1
ENSGALG00000001490	Uncharacterised
ENSGALG00000002041	agrin
ENSGALG00000002757	Uncharacterised
ENSGALG00000002854	Uncharacterised
ENSGALG00000003115	Uncharacterised
ENSGALG00000003473	secreted frizzled-related protein 1
ENSGALG00000004322	Uncharacterised
ENSGALG00000004414	leucine zipper protein 2
ENSGALG00000004448	family with sequence similarity 5, member B
ENSGALG00000004630	similar to cHz-cadherin
ENSGALG00000004814	rhophilin, Rho GTPase binding protein 2
ENSGALG00000005259	vasoactive intestinal peptide receptor 1
ENSGALG00000005956	annexin A8-like 1
ENSGALG00000006269	Uncharacterised
ENSGALG00000006306	urocanase domain containing 1
ENSGALG00000006313	interleukin 4 receptor
ENSGALG00000007211	cadherin-like 22
ENSGALG00000007226	osteocrin
ENSGALG00000007367	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2
ENSGALG00000007410	similar to hDDM36
ENSGALG00000007487	chromosome 21 open reading frame 58
ENSGALG00000007596	hypothetical LOC416086
ENSGALG00000008150	RAS protein activator like 1 (GAP1 like)
ENSGALG00000008263	contactin 4
ENSGALG00000008874	solute carrier family 13 (sodium/sulfate symporters), member 1
ENSGALG00000008926	Ca2+-dependent activator protein for secretion 2
ENSGALG00000008980	von Willebrand factor A domain containing 2
ENSGALG00000009006	six transmembrane epithelial antigen of the prostate 1
ENSGALG00000009315	Uncharacterised
ENSGALG00000009497	arginine vasopressin receptor 2 (nephrogenic diabetes insipidus)
ENSGALG00000009515	guanine nucleotide binding protein (G protein), gamma 11
ENSGALG00000009684	G protein-coupled receptor 26

ENSGALG00000009799	Meis homeobox 2
ENSGALG00000010035	nuclear receptor subfamily 3, group C, member 2
ENSGALG00000011717	hypothetical LOC417937
ENSGALG00000011813	HEG homolog 1 (zebrafish)
ENSGALG00000011836	solute carrier family 6 (proline IMINO transporter), member 20
ENSGALG00000011858	potassium voltage-gated channel, subfamily H (eag-related), member 5
ENSGALG00000012163	brain-derived neurotrophic factor
ENSGALG00000012183	neuronal pentraxin receptor
ENSGALG00000012235	neurogenic differentiation 6
ENSGALG00000012421	Rho GTPase activating protein 15
ENSGALG00000012568	TIMP metallopeptidase inhibitor 3)
ENSGALG00000012908	solute carrier family 13 (sodium/sulfate symporters), member 4
ENSGALG00000013154	solute carrier organic anion transporter family, member 1C1
ENSGALG00000013168	islet amyloid polypeptide
ENSGALG00000013515	solute carrier family 4, sodium bicarbonate cotransporter, member 5; similar to sodium bicarbonate cotransporter-like protein
ENSGALG00000014414	gamma-aminobutyric acid (GABA) receptor, rho 3
ENSGALG00000014634	silver homolog (mouse)
ENSGALG00000014978	IQ motif containing GTPase activating protein 2
ENSGALG00000015205	tyrosinase-related protein 1
ENSGALG00000015720	chondrolectin
ENSGALG00000016411	similar to collagen XIV; collagen, type XIV, alpha 1 (undulin); similar to collagen, type XIV, alpha 1 (undulin)
ENSGALG00000016616	similar to Kallmann syndrome gene product;
ENSGALG00000016884	solute carrier family 15 (oligopeptide transporter), member 1
ENSGALG00000017343	folate receptor 1 (adult)
ENSGALG00000018557	similar to extracellular-superoxide dismutase (EC 1.15.1.1); superoxide dismutase 3, extracellular
ENSGALG00000023051	Uncharacterised
ENSGALG00000023580	Uncharacterised

Specific pre-hatching B responsive genes

Ensembl ID	Description
ENSGALG00000001396	serpin peptidase inhibitor, clade D (heparin cofactor), member 1
ENSGALG00000001696	S-antigen; retina and pineal gland (arrestin)
ENSGALG00000002223	LIM homeobox 9
ENSGALG00000003842	growth hormone releasing hormone
ENSGALG00000003895	PR domain containing 12
ENSGALG00000007025	copine VIII
ENSGALG00000007588	glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa)
ENSGALG00000007908	EGF-containing fibulin-like extracellular matrix protein 1

ENSGALG00000008188	tripartite motif-containing 36
ENSGALG00000008883	transcription factor 7-like 2 (T-cell specific, HMG-box)
ENSGALG00000009129	distal-less homeobox 5
ENSGALG00000009737	tachykinin, precursor 1 (substance K, substance P, neurokinin 1, neurokinin 2, neuromedin L, neurokinin alpha, neuropeptide K, neuropeptide gamma)
ENSGALG00000009739	adhesion molecule with Ig-like domain 2
ENSGALG00000009740	similar to RAS guanyl releasing protein 1 (calcium and DAG-regulated); RAS guanyl releasing protein 1 (calcium and DAG-regulated)
ENSGALG00000010865	transmembrane protein 196
ENSGALG00000012907	melanocortin 4 receptor
ENSGALG00000012911	synaptotagmin X
ENSGALG00000013294	cytochrome P450, family 19, subfamily A, polypeptide 1
ENSGALG00000013890	melanocortin 5 receptor
ENSGALG00000014233	fibulin 1
ENSGALG00000015143	transthyretin
ENSGALG00000015419	proenkephalin
ENSGALG00000015529	Wolfram syndrome 1 (wolframin)
ENSGALG00000016035	GFR receptor alpha 4; similar to GFR receptor alpha 4
ENSGALG00000016324	glutathione S-transferase alpha 3
ENSGALG00000017418	neuronal pentraxin I
ENSGALG00000019277	solute carrier organic anion transporter family, member 1B3
ENSGALG00000020381	deiodinase, iodothyronine, type III
ENSGALG00000022819	Purkinje cell protein 4

Interacting pre- and post-hatching B responsive genes: "cumulative effect"

Ensembl ID	Description
ENSGALG00000000733	myosin VIIA
ENSGALG00000001006	tumor protein p73
ENSGALG00000001063	PR domain containing 16
ENSGALG00000002389	integrin, beta 4
ENSGALG00000004879	solute carrier family 6 (neurotransmitter transporter, GABA), member 11
ENSGALG00000005400	calcium channel, voltage-dependent, alpha 2/delta 3 subunit
ENSGALG00000005985	growth differentiation factor 10
ENSGALG00000006325	similar to netrin 4
ENSGALG00000006413	KIAA1199
ENSGALG00000006449	glutamate receptor interacting protein 2
ENSGALG00000008445	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3
ENSGALG00000008465	sortilin-related VPS10 domain containing receptor 1
ENSGALG00000009034	anaplastic lymphoma kinase (Ki-1)
ENSGALG00000009589	glutamate decarboxylase 1 (brain, 67kDa)

ENSGALG00000010781	glycine receptor, alpha 3
ENSGALG00000010858	low density lipoprotein-related protein 2
ENSGALG00000012917	cadherin 6, type 2, K-cadherin (fetal kidney); similar to CDH6 protein
ENSGALG00000013953	tachykinin receptor 1
ENSGALG00000015673	zinc finger homeodomain 4
ENSGALG00000015857	carbonic anhydrase III, muscle specific
ENSGALG00000015865	similar to C6orf37
ENSGALG00000016017	solute carrier family 4, sodium borate transporter, member 11
ENSGALG00000016577	otoferlin
ENSGALG00000016866	fibroblast growth factor 14
ENSGALG00000017021	ATPase, Cu++ transporting, beta polypeptide
ENSGALG00000017040	Uncharacterised
ENSGALG00000017068	klotho
ENSGALG00000017405	Uncharacterised
ENSGALG00000021039	hexokinase domain containing 1
ENSGALG00000023552	Uncharacterised

(b) Hypothalamus

Pre- and post-hatching B responsive genes

Ensembl ID	Description
ENSGALG00000001136	similar to enhancer of split related protein-7
ENSGALG00000001896	netrin G1
ENSGALG00000002111	SEC14-like 5 (S. cerevisiae)
ENSGALG00000002331	calbindin 2, 29kDa (calretinin)
ENSGALG00000005526	hairy and enhancer of split 6 (Drosophila)
ENSGALG00000006271	Rac GTPase activating protein 1
ENSGALG00000006838	similar to iron binding protein
ENSGALG00000007772	cerebellin 4 precursor
ENSGALG00000008306	fibrinogen-like 2
ENSGALG00000009058	ectonucleoside triphosphate diphosphohydrolase 2
ENSGALG00000009791	prospero-related homeobox 1
ENSGALG00000009861	retinal degeneration 3
ENSGALG00000010065	potassium channel, subfamily K, member 5
ENSGALG00000010461	early B-cell factor 3
ENSGALG00000010583	vitrin
ENSGALG00000011066	calmin (calponin-like, transmembrane)
ENSGALG00000011127	B-cell CLL/lymphoma 11B (zinc finger protein)
ENSGALG00000011940	cholecystokinin
ENSGALG00000012732	phosphatase and actin regulator 1

Specific pre-hatching B responsive genes

Ensembl ID	Description
ENSGALG00000001063	PR domain containing 16
ENSGALG00000003149	inositol 1,4,5-triphosphate receptor, type 3
ENSGALG00000003895	PR domain containing 12
ENSGALG00000004860	RAS, dexamethasone-induced 1
ENSGALG00000006014	protein kinase C, beta
ENSGALG00000007113	Uncharacterised
ENSGALG00000009095	luteinizing hormone/choriogonadotropin receptor
ENSGALG00000010402	prostaglandin-D synthase
ENSGALG00000013193	iroquois homeobox 2
ENSGALG00000014484	Uncharacterised
ENSGALG00000014843	tumor protein D52-like 1
ENSGALG00000015824	glycoprotein hormones, alpha polypeptide
ENSGALG00000015857	carbonic anhydrase III, muscle specific
ENSGALG00000017044	transient receptor potential cation channel, subfamily C,

Specific post-hatching B responsive genes

Ensembl ID	Description
ENSGALG00000000098	anthrax toxin receptor 1
ENSGALG00000000168	adenosine A1 receptor
ENSGALG00000000376	Uncharacterised
ENSGALG00000000694	Uncharacterised
ENSGALG00000000695	major facilitator superfamily domain containing 4
ENSGALG00000000820	5-hydroxytryptamine (serotonin) receptor 1D
ENSGALG00000001227	Uncharacterised
ENSGALG00000001282	gamma-aminobutyric acid (GABA) A receptor, delta
ENSGALG00000001505	neuronal guanine nucleotide exchange factor
ENSGALG00000002260	cytokine inducible SH2-containing protein
ENSGALG00000002470	cytochrome P450, family 27, subfamily A, polypeptide 1
ENSGALG00000003285	protocadherin 24
ENSGALG00000003437	Uncharacterised
ENSGALG00000003670	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B
ENSGALG00000004011	Uncharacterised
ENSGALG00000004074	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1
ENSGALG00000004270	aldehyde dehydrogenase 1 family, member A2
ENSGALG00000004838	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)
ENSGALG00000005258	somatostatin receptor 5
ENSGALG00000005657	corticotropin releasing hormone receptor 2

ENSGALG00000005721	diacylglycerol kinase, gamma 90kDa
ENSGALG00000005752	ATP-binding cassette, sub-family A (ABC1), member 4
ENSGALG00000005853	5-hydroxytryptamine (serotonin) receptor 2C
ENSGALG00000006008	homer homolog 2 (Drosophila)
ENSGALG00000006021	calcium channel, voltage-dependent, gamma subunit 3
ENSGALG00000006439	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6
ENSGALG00000006886	dachshund homolog 2 (Drosophila)
ENSGALG00000007004	5-hydroxytryptamine (serotonin) receptor 3A
ENSGALG00000007141	leucine-rich repeat kinase 1
ENSGALG00000007184	FEZ family zinc finger 2
ENSGALG00000007278	glutamate receptor, ionotropic, N-methyl D-aspartate 2A
ENSGALG00000007349	RAS-like, family 12
ENSGALG00000007415	SH3 domain containing ring finger 2
ENSGALG00000008032	G protein-coupled receptor 22
ENSGALG00000008135	SATB homeobox 2
ENSGALG00000008308	basic helix-loop-helix domain containing, class B, 2
ENSGALG00000008631	TYRO3 protein tyrosine kinase
ENSGALG00000008671	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6
ENSGALG00000008940	spectrin, beta, non-erythrocytic 5
ENSGALG00000009252	phospholipase D1, phosphatidylcholine-specific
ENSGALG00000009859	TBC1 domain family, member 30
ENSGALG00000010705	zinc finger protein 238
ENSGALG00000010801	transmembrane protein 61
ENSGALG00000011122	Uncharacterised
ENSGALG00000011254	SATB homeobox 1
ENSGALG00000011406	netrin 4
ENSGALG00000011592	muscle RAS oncogene homolog
ENSGALG00000012046	similar to ARPP-21 protein
ENSGALG00000012054	doublecortin-like kinase 3
ENSGALG00000012154	F-box protein 34
ENSGALG00000012235	neurogenic differentiation 6
ENSGALG00000012254	potassium inwardly-rectifying channel, subfamily J, member 4
ENSGALG00000012322	potassium channel tetramerisation domain containing 16
ENSGALG00000012367	tripartite motif-containing 9
ENSGALG00000012542	RASD family, member 2
ENSGALG00000012890	diacylglycerol kinase, iota
ENSGALG00000013051	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A

ENSGALG00000013795	human immunodeficiency virus type I enhancer binding protein 2
ENSGALG00000013948	RAS-like, family 11, member B
ENSGALG00000014011	lymphoid-restricted membrane protein
ENSGALG00000014186	metallophosphoesterase domain containing 1
ENSGALG00000014812	SID1 transmembrane family, member 1
ENSGALG00000014907	discoidin, CUB and LCCL domain containing 1
ENSGALG00000015271	filamin A interacting protein 1-like
ENSGALG00000015403	EPH receptor A3
ENSGALG00000015626	regulator of G-protein signalling 12
ENSGALG00000015970	collagen, type IX, alpha 1
ENSGALG00000016084	R-spondin 2 homolog (Xenopus laevis)
ENSGALG00000016095	empty spiracles homeobox 1
ENSGALG00000016155	collagen, type XIX, alpha 1; hypothetical protein LOC772348
ENSGALG00000016391	connector enhancer of kinase suppressor of Ras 2
ENSGALG00000016396	collectin sub-family member 11
ENSGALG00000016744	gamma-aminobutyric acid (GABA) A receptor, alpha 5
ENSGALG00000016843	collagen, type IV, alpha 2
ENSGALG00000016920	LIM domain 7
ENSGALG00000016944	protocadherin 8
ENSGALG00000016983	Uncharacterised
ENSGALG00000017378	cartilage acidic protein 1
ENSGALG00000017690	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 2
ENSGALG00000018942	neurogranin (protein kinase C substrate, RC3)
ENSGALG00000019842	transcription factor AP-2delta
ENSGALG00000020975	Uncharacterised
ENSGALG00000022001	hypothetical LOC415928
ENSGALG00000022782	Uncharacterised
ENSGALG00000022988	Uncharacterised
ENSGALG00000023441	reticulon 4 receptor-like 2
ENSGALG00000023881	plexin domain containing 1
ENSGALG00000024111	Uncharacterised

Table A7. Lists of down- and up-regulated genes (highlighted in green and red, respectively) in the (a) hippocampus and (b) hypothalamus submitted to Ingenuity Pathway Analysis (IPA) after filtering the Vector Analysis data according to the behavioural categories as described in full detail in Chapter 4 (Section 4.3.13.1). The genes highlighted in black are the non-redundant "focus" genes with records in the IPA server, whilst in blue are the "non-focus" genes.

(a) Hippocampus

Pre- and post-hatching B responsive genes

Ensembl ID	Symbol	Description
ENSGALG00000000713	ZFHX3	zinc finger homeobox 3
ENSGALG00000000745	SLC26A9	solute carrier family 26, member 9
ENSGALG00000001115	MMEL1	membrane metallo-endopeptidase-like 1
ENSGALG00000002041	AGRN	agrin
ENSGALG00000003115	COL4A3	collagen, type IV, alpha 3 (Goodpasture antigen)
ENSGALG00000003473	SFRP1	secreted frizzled-related protein 1
ENSGALG00000004448	FAM5B	family with sequence similarity 5, member B
ENSGALG00000004814	RHPN2	rhophilin, Rho GTPase binding protein 2
ENSGALG00000005259	VIPR1	vasoactive intestinal peptide receptor 1
ENSGALG00000006269	TMEM72	transmembrane protein 72
ENSGALG00000006306	UROC1	urocanate hydratase 1
ENSGALG00000007211	CDH22	cadherin 22, type 2
ENSGALG00000007226	OSTN	osteocrin
ENSGALG00000007367	WFIKKN2	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2
ENSGALG00000007410	IGDCC4	immunoglobulin superfamily, DCC subclass, member 4
ENSGALG00000007487	C21orf58	chromosome 21 open reading frame 58
ENSGALG00000008263	CNTN4	contactin 4
ENSGALG00000008874	SLC13A1	solute carrier family 13 (sodium/sulfate symporters), member 1
ENSGALG00000008980	VWA2	von Willebrand factor A domain containing 2
ENSGALG00000009006	STEAP1	six transmembrane epithelial antigen of the prostate 1
ENSGALG00000009497	AVPR2	arginine vasopressin receptor 2
ENSGALG00000009515	GNG11	guanine nucleotide binding protein (G protein), gamma 11
ENSGALG00000009684	GPR26	G protein-coupled receptor 26
ENSGALG00000009799	MEIS2	Meis homeobox 2
ENSGALG00000011717	2010107G12Rik	RIKEN cDNA 2010107G12 gene
ENSGALG00000011858	KCNH5	potassium voltage-gated channel, subfamily H (eag-related), member 5
ENSGALG00000012568	TIMP3	TIMP metallopeptidase inhibitor 3

ENSGALG00000012908	SLC13A4	solute carrier family 13 (sodium/sulfate symporters), member 4
ENSGALG00000013154	SLCO1C1	solute carrier organic anion transporter family, member 1C1
ENSGALG00000013515	SLC4A5	solute carrier family 4, sodium bicarbonate cotransporter, member 5
ENSGALG00000014634	PMEL	premelanosome protein
ENSGALG00000015205	TYRP1	tyrosinase-related protein 1
ENSGALG00000016411	COL14A1	collagen, type XIV, alpha 1
ENSGALG00000016616	KAL1	Kallmann syndrome 1 sequence
ENSGALG00000016884	SLC15A1	solute carrier family 15 (oligopeptide transporter), member 1
ENSGALG00000017343	FOLR1	folate receptor 1 (adult)
ENSGALG00000018557	SOD3	superoxide dismutase 3, extracellular
ENSGALG00000023580	CLDN19	claudin 19
ENSGALG00000002757	PCDH15	protocadherin-related 15
ENSGALG00000004414	LUZP2	leucine zipper protein 2
ENSGALG00000008926	CADPS2	Ca++-dependent secretion activator 2
ENSGALG00000010035	NR3C2	nuclear receptor subfamily 3, group C, member 2
ENSGALG00000012163	BDNF	brain-derived neurotrophic factor
ENSGALG00000012183	Npcd	neuronal pentraxin chromo domain
ENSGALG00000012235	NEUROD6	neuronal differentiation 6
ENSGALG00000012421	ARHGAP15	Rho GTPase activating protein 15
ENSGALG00000014414	Gabrr3	gamma-aminobutyric acid (GABA) receptor, rho 3
ENSGALG00000015720	CHODL	chondrolectin

Specific pre-hatching B responsive genes

Ensembl ID	Symbol	Description
ENSGALG00000001396	SERPIND1	serpin peptidase inhibitor, clade D (heparin cofactor), member 1
ENSGALG00000001696	SAG	S-antigen; retina and pineal gland (arrestin)
ENSGALG00000002223	LHX9	LIM homeobox 9
ENSGALG00000003895	PRDM12	PR domain containing 12
ENSGALG00000007025	CPNE8	copine VIII
ENSGALG00000007588	GAD2	glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa)
ENSGALG00000007908	EFEMP1	EGF containing fibulin-like extracellular matrix protein 1
ENSGALG00000008188	TRIM36	tripartite motif containing 36
ENSGALG00000008883	TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)
ENSGALG00000009129	DLX5	distal-less homeobox 5
ENSGALG00000009737	TAC1	tachykinin, precursor 1
ENSGALG00000009739	AMIGO2	adhesion molecule with Ig-like domain 2
ENSGALG00000009740	RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)
ENSGALG00000010865	TMEM196	transmembrane protein 196

ENSGALG00000012907	MC4R	melanocortin 4 receptor
ENSGALG00000012911	SYT10	synaptotagmin X
ENSGALG00000013294	CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1
ENSGALG00000013890	MC5R	melanocortin 5 receptor
ENSGALG00000014233	FBLN1	fibulin 1
ENSGALG00000015143	TTR	transthyretin
ENSGALG00000015419	PENK	proenkephalin
ENSGALG00000015529	WFS1	Wolfram syndrome 1 (wolframin)
ENSGALG00000016324	Gsta3	glutathione S-transferase, alpha 3
ENSGALG00000019277	SLCO1B1	solute carrier organic anion transporter family, member 1B1
ENSGALG00000017418	NPTX1	neuronal pentraxin I

Interacting pre- and post-hatching B responsive genes: "cumulative effect"

Ensembl ID	Symbol	Description
ENSGALG00000000733	MYO7A	myosin VIIA
ENSGALG00000001006	TP73	tumor protein p73
ENSGALG00000001063	PRDM16	PR domain containing 16
ENSGALG00000002389	ITGB4	integrin, beta 4
ENSGALG00000004879	SLC6A11	solute carrier family 6 (neurotransmitter transporter, GABA), member 11
ENSGALG00000005400	CACNA2D3	calcium channel, voltage-dependent, alpha 2/delta subunit 3
ENSGALG00000006413	KIAA1199	KIAA1199
ENSGALG00000006449	GRIP2	glutamate receptor interacting protein 2
ENSGALG00000008445	SLC24A3	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3
ENSGALG00000008465	SORCS1	sortilin-related VPS10 domain containing receptor 1
ENSGALG00000009034	ALK	anaplastic lymphoma receptor tyrosine kinase
ENSGALG00000009589	GAD1	glutamate decarboxylase 1 (brain, 67kDa)
ENSGALG00000010781	GLRA3	glycine receptor, alpha 3
ENSGALG00000010858	LRP2	low density lipoprotein receptor-related protein 2
ENSGALG00000012917	CDH6	cadherin 6, type 2, K-cadherin (fetal kidney)
ENSGALG00000013953	TACR1	tachykinin receptor 1
ENSGALG00000015673	ZFHX4	zinc finger homeobox 4
ENSGALG00000015865	FAM46A	family with sequence similarity 46, member A
ENSGALG00000016017	SLC4A11	solute carrier family 4, sodium borate transporter, member 11
ENSGALG00000016577	OTOF	otoferlin
ENSGALG00000017021	ATP7B	ATPase, Cu++ transporting, beta polypeptide
ENSGALG00000017068	KL	klotho
ENSGALG00000017405	NPR3	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)
ENSGALG00000021039	HKDC1	hexokinase domain containing 1

ENSGALG00000005985	GDF10	growth differentiation factor 10
ENSGALG00000015857	CA3	carbonic anhydrase III, muscle specific

(b) Hypothalamus

Pre- and post-hatching B responsive genes

Ensembl ID	Symbol	Description
ENSGALG00000001896	NTNG1	netrin G1
ENSGALG00000002331	CALB2	calbindin 2
ENSGALG00000007772	CBLN4	cerebellin 4 precursor
ENSGALG00000009791	PROX1	prospero homeobox 1
ENSGALG00000011940	CCK	cholecystokinin
ENSGALG00000002111	SEC14L5	SEC14-like 5 (S. cerevisiae)
ENSGALG00000006271	RACGAP1	Rac GTPase activating protein 1
ENSGALG00000008306	FGL2	fibrinogen-like 2
ENSGALG00000009058	ENTPD2	ectonucleoside triphosphate diphosphohydrolase 2
ENSGALG00000009861	RD3	retinal degeneration 3
ENSGALG00000010065	KCNK5	potassium channel, subfamily K, member 5
ENSGALG00000012732	PHACTR1	phosphatase and actin regulator 1

Specific pre-hatching B responsive genes

Ensembl ID	Symbol	Description
ENSGALG00000009095	LHCGR	luteinizing hormone/choriogonadotropin receptor
ENSGALG00000010402	HPGDS	hematopoietic prostaglandin D synthase
ENSGALG00000013193	IRX2	iroquois homeobox 2
ENSGALG00000015824	CGA	glycoprotein hormones, alpha polypeptide
ENSGALG00000001063	PRDM16	PR domain containing 16
ENSGALG00000003149	ITPR3	inositol 1,4,5-trisphosphate receptor, type 3
ENSGALG00000003895	PRDM12	PR domain containing 12
ENSGALG00000004860	RASD1	RAS, dexamethasone-induced 1
ENSGALG00000006014	PRKCB	protein kinase C, beta
ENSGALG00000014843	TPD52L1	tumor protein D52-like 1
ENSGALG00000015857	CA3	carbonic anhydrase III, muscle specific
ENSGALG00000017044	TRPC4	transient receptor potential cation channel, subfamily C, member 4

Specific post-hatching B responsive genes

Ensembl ID	Symbol	Description
ENSGALG00000004838	BUB1B	BUB1 mitotic checkpoint serine/threonine kinase B
ENSGALG00000008671	ST8SIA6	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6
ENSGALG00000013051	SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A
ENSGALG00000000098	ANTXR1	anthrax toxin receptor 1
ENSGALG00000000168	ADORA1	adenosine A1 receptor
ENSGALG00000000694	FMNL1	formin-like 1
ENSGALG00000000695	MFSD4	major facilitator superfamily domain containing 4
ENSGALG00000000820	HTR1D	5-hydroxytryptamine (serotonin) receptor 1D, G protein-coupled

ENSGALG00000001227	PIK3R6	phosphoinositide-3-kinase, regulatory subunit 6
ENSGALG00000001282	GABRD	gamma-aminobutyric acid (GABA) A receptor, delta
ENSGALG00000001505	NGEF	neuronal guanine nucleotide exchange factor
ENSGALG00000002260	CISH	cytokine inducible SH2-containing protein
ENSGALG00000002470	CYP27A1	cytochrome P450, family 27, subfamily A, polypeptide 1
ENSGALG00000003285	CDHR2	cadherin-related family member 2
ENSGALG00000003670	MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)
ENSGALG00000004074	KCNS1	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1
ENSGALG00000004270	ALDH1A2	aldehyde dehydrogenase 1 family, member A2
ENSGALG00000005258	SSTR5	somatostatin receptor 5
ENSGALG00000005657	CRHR2	corticotropin releasing hormone receptor 2
ENSGALG00000005721	DGKG	diacylglycerol kinase, gamma 90kDa
ENSGALG00000005752	ABCA4	ATP-binding cassette, sub-family A (ABC1), member 4
ENSGALG00000005853	HTR2C	5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled
ENSGALG00000006008	HOMER2	homer homolog 2 (Drosophila)
ENSGALG00000006021	CACNG3	calcium channel, voltage-dependent, gamma subunit 3
ENSGALG00000006439	ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6
ENSGALG00000006886	DACH2	dachshund homolog 2 (Drosophila)
ENSGALG00000007004	HTR3A	5-hydroxytryptamine (serotonin) receptor 3A, ionotropic
ENSGALG00000007141	LRRK1	leucine-rich repeat kinase 1
ENSGALG00000007184	FEZF2	FEZ family zinc finger 2
ENSGALG00000007278	GRIN2A	glutamate receptor, ionotropic, N-methyl D-aspartate 2A
ENSGALG00000007349	RASL12	RAS-like, family 12
ENSGALG00000008032	GPR22	G protein-coupled receptor 22
ENSGALG00000008135	SATB2	SATB homeobox 2
ENSGALG00000008308	BHLHE40	basic helix-loop-helix family, member e40
ENSGALG00000008631	TYRO3	TYRO3 protein tyrosine kinase
ENSGALG00000008940	SPTBN5	spectrin, beta, non-erythrocytic 5
ENSGALG00000009859	TBC1D30	TBC1 domain family, member 30
ENSGALG00000010705	ZBTB18	zinc finger and BTB domain containing 18
ENSGALG00000010801	TMEM61	transmembrane protein 61
ENSGALG00000011254	SATB1	SATB homeobox 1
ENSGALG00000011406	NTN4	netrin 4
ENSGALG00000011592	MRAS	muscle RAS oncogene homolog
ENSGALG00000012046	ARPP21	cAMP-regulated phosphoprotein, 21kDa
ENSGALG00000012054	DCLK3	doublecortin-like kinase 3
ENSGALG00000012154	FBXO34	F-box protein 34

ENSGALG00000012235	NEUROD6	neuronal differentiation 6
ENSGALG00000012254	KCNJ4	potassium inwardly-rectifying channel, subfamily J, member 4
ENSGALG00000012322	KCTD16	potassium channel tetramerisation domain containing 16
ENSGALG00000012542	RASD2	RASD family, member 2
ENSGALG00000012890	DGKI	diacylglycerol kinase, iota
ENSGALG00000013948	RASL11B	RAS-like, family 11, member B
ENSGALG00000014011	LRMP	lymphoid-restricted membrane protein
ENSGALG00000014186	MPPED1	metallophosphoesterase domain containing 1
ENSGALG00000014812	SIDT1	SID1 transmembrane family, member 1
ENSGALG00000014907	DCBLD1	discoidin, CUB and LCCL domain containing 1
ENSGALG00000015271	FILIP1L	filamin A interacting protein 1-like
ENSGALG00000015403	EPHA3	EPH receptor A3
ENSGALG00000015626	RGS12	regulator of G-protein signaling 12
ENSGALG00000015970	COL9A1	collagen, type IX, alpha 1
ENSGALG00000016084	RSPO2	R-spondin 2
ENSGALG00000016095	EMX1	empty spiracles homeobox 1
ENSGALG00000016391	CNKSR2	connector enhancer of kinase suppressor of Ras 2
ENSGALG00000016396	COLEC11	collectin sub-family member 11
ENSGALG00000016744	GABRA5	gamma-aminobutyric acid (GABA) A receptor, alpha 5
ENSGALG00000016843	COL4A2	collagen, type IV, alpha 2
ENSGALG00000016920	LMO7	LIM domain 7
ENSGALG00000016944	PCDH8	protocadherin 8
ENSGALG00000017378	CRTAC1	cartilage acidic protein 1
ENSGALG00000017690	KCNS2	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 2
ENSGALG00000022001	CAMKV	CaM kinase-like vesicle-associated
ENSGALG00000023441	RTN4RL2	reticulon 4 receptor-like 2
ENSGALG00000024111	FNDC9	fibronectin type III domain containing 9

Table A8. Significant functional biological categories identified by the Ingenuity Pathways Analysis (IPA) performed using the gene lists after filtering the Vector Analysis data with specific behavioural categories (full details in Chapter 4, paragraph 4.3.13.1 for details) in the (a) hippocampus and (b) hypothalamus.

(a) Hippocampus

Pre- and post-hatching B responsive genes

Category	p-value	Genes
Renal and Urological Disease	1.89E-05-2.73E-02	SLC4A5,TIMP3,AVPR2,CLDN19,COL4A3, NR3C2,SFRP1,SLC13A1
Cellular Function and Maintenance	3.67E-05-2.48E-02	SLC4A5,NEUROD6,SLC26A9,BDNF, CNTN4,CLDN19,PCDH15,CADPS2,NR3C2,AGRN
Cardiovascular System Development	8.35E-05-2.09E-02	SLC4A5,TIMP3,AVPR2,BDNF, COL4A3,NR3C2,SOD3
Hematological System Development	8.35E-05-2.73E-02	SLC4A5,TIMP3,AVPR2,VIPR1,BDNF, COL4A3,NR3C2,SFRP1,AGRN,SOD3
Cell-To-Cell Signaling and Interaction	1.28E-04-2.73E-02	TIMP3,VIPR1,BDNF,CNTN4,CLDN19, NR3C2,CADPS2,SFRP1,AGRN
Cellular Assembly and Organization	1.28E-04-2.48E-02	NEUROD6,BDNF,CNTN4,CLDN19, PCDH15,CADPS2,NR3C2,SFRP1,AGRN
Nervous System Development and Function	1.28E-04-2.97E-02	SLC4A5,NEUROD6,BDNF,CNTN4,CLDN19,KAL1,PCDH15,CADPS2,SOD3,FOLR1,VIPR1, NR3C2,SFRP1,AGRN
Tissue Development	1.28E-04-2.9E-02	NEUROD6,TIMP3,COL14A1,BDNF,MMEL1,CLDN19, CNTN4,COL4A3,CADPS2,FOLR1, SFRP1,AGRN,ZFHX3
Cellular Movement	1.35E-04-2.9E-02	TIMP3,VIPR1,BDNF,COL4A3,CNTN4,KAL1,CADPS2, NR3C2,SFRP1,SOD3,FOLR1
Immune Cell Trafficking	1.35E-04-3.14E-03	TIMP3,VIPR1,COL4A3,NR3C2,SFRP1,SOD3
Inflammatory Response	1.35E-04-1.5E-02	PMEL,TIMP3,VIPR1,COL4A3,NR3C2,SFRP1,SOD3
Molecular Transport	1.92E-04-2.95E-02	SLC4A5,TIMP3,SLCO1C1,SLC26A9,BDNF,SLC15A1, COL4A3,SLC13A4,NR3C2,CADPS2,SLC13A1,FOLR1
Cellular Development	4.7E-04-2.73E-02	TIMP3,NEUROD6,BDNF,MMEL1,COL4A3,CNTN4, WFIKKN2,MEIS2,PCDH15,CADPS2,SOD3,FOLR1, TYRP1,NR3C2,SFRP1,AGRN,ZFHX3
Behavior	5.48E-04-2.73E-02	SLC4A5,TIMP3,BDNF,CLDN19,PCDH15, NR3C2,CADPS2,GPR26,SOD3
Connective Tissue Disorders	7.43E-04-5.01E-03	TIMP3,COL14A1,COL4A3
Tissue Morphology	8.15E-04-2.73E-02	BDNF,CNTN4,NR3C2,CADPS2,AGRN,SOD3,FOLR1
Cell Morphology	8.39E-04-2.73E-02	SLC4A5,NEUROD6,TIMP3,SLC26A9,BDNF, CNTN4,COL4A3,PCDH15,CADPS2,NR3C2,AGRN
Amino Acid Metabolism	9.15E-04-2.73E-02	PMEL,TYRP1,SLCO1C1,BDNF,FOLR1
Hair and Skin Development and Function	9.15E-04-9.99E-03	TYRP1,PMEL,TIMP3,BDNF

Small Molecule Biochemistry	9.15E-04-2.87E-02	PMEL,TYRP1,SLC4A5,TIMP3,SLCO1C1,BDNF, COL4A3,NR3C2,CADPS2,SLC13A1,FOLR1
Reproductive System Disease	1.5E-03-1.5E-02	KAL1,NR3C2,SLC13A1
Cardiovascular Disease	2.12E-03-2.41E-02	TIMP3,AVPR2,BDNF,NR3C2
Auditory Disease	2.51E-03-9.1E-03	BDNF,COL4A3,PCDH15
Auditory and Vestibular System Development and Function	2.51E-03-9.99E-03	BDNF,FOLR1
Carbohydrate Metabolism	2.51E-03-9.99E-03	TIMP3,SLCO1C1,BDNF
Cell Cycle	2.51E-03-7.51E-03	MEIS2,ZFHX3
Cell Death and Survival	2.51E-03-2.24E-02	BDNF,SFRP1,AGRN,SOD3
Cellular Compromise	2.51E-03-2.73E-02	TYRP1,BDNF
Cellular Growth and Proliferation	2.51E-03-2.48E-02	TIMP3,BDNF,WFIKKN2,SFRP1,AGRN,ZFHX3
Dermatological Diseases and Conditions	2.51E-03-2.24E-02	TYRP1,PMEL,COL14A1,BDNF,COL4A3,CLDN19
Developmental Disorder	2.51E-03-1.99E-02	TYRP1,NEUROD6,VIPR1,BDNF,KAL1,SFRP1, SLC13A1,AGRN,FOLR1
Drug Metabolism	2.51E-03-2.73E-02	SLCO1C1,SLC15A1,FOLR1
Embryonic Development	2.51E-03-2.9E-02	TIMP3,NEUROD6,MMEL1,BDNF,CADPS2, SFRP1,AGRN,ZFHX3,FOLR1
Endocrine System Development and Function	2.51E-03-2.24E-02	SLC4A5,SLCO1C1,BDNF,CADPS2,SLC13A1
Endocrine System Disorders	2.51E-03-1.74E-02	AVPR2,BDNF,KAL1,NR3C2
Gastrointestinal Disease	2.51E-03-2.24E-02	SLCO1C1,TIMP3,COL14A1,AVPR2,BDNF, COL4A3
Hematological Disease	2.51E-03-2.73E-02	AVPR2,COL4A3,CLDN19,NR3C2,SLC13A1
Hereditary Disorder	2.51E-03-1.99E-02	TYRP1,TIMP3,AVPR2,COL14A1,BDNF,CLDN19, COL4A3,KAL1,PCDH15,NR3C2,FOLR1
Immunological Disease	2.51E-03-5.01E-03	COL4A3
Inflammatory Disease	2.51E-03-1.5E-02	COL4A3
Lipid Metabolism	2.51E-03-2.87E-02	SLC4A5,TYRP1,SLCO1C1,BDNF,COL4A3,SLC13A1
Metabolic Disease	2.51E-03-2.73E-02	SLC4A5,TYRP1,AVPR2,BDNF,COL4A3,NR3C2, SLC13A1
Neurological Disease	2.51E-03-2.48E-02	AVPR2,RHPN2,BDNF,COL4A3,KAL1,PCDH15, CADPS2,SLC13A1,SFRP1,AGRN,FOLR1
Nutritional Disease	2.51E-03-5.01E-03	AVPR2,BDNF
Ophthalmic Disease	2.51E-03-7.51E-03	TYRP1,TIMP3,CLDN19,PCDH15,CADPS2

Organ Development	2.51E-03-2.24E-02	NEUROD6,MMEL1,BDNF,ZFHX3
Organ Morphology	2.51E-03-2.73E-02	SLC4A5,NEUROD6,BDNF,CADPS2,FOLR1
Organismal Development	2.51E-03-2.9E-02	NEUROD6,TIMP3,MMEL1,BDNF,COL4A3,SFRP1, ZFHX3,FOLR1
Organismal Functions	2.51E-03-2.24E-02	TIMP3,BDNF,COL4A3,NR3C2
Organismal Injury and Abnormalities	2.51E-03-2.48E-02	TYRP1,TIMP3,COL14A1,AVPR2,BDNF,COL4A3, NR3C2,SOD3
Psychological Disorders	2.51E-03-1.5E-02	BDNF
Respiratory Disease	2.51E-03-1.99E-02	BDNF,KAL1
Skeletal and Muscular System Development and Function	2.51E-03-2.7E-02	BDNF,WFIKKN2,ZFHX3
Hepatic System Disease	3.08E-03-2.24E-02	SLCO1C1,TIMP3,AVPR2
Cancer	5.01E-03-2.7E-02	PMEL,TIMP3,RHPN2,GNG11,BDNF,NR3C2,SFRP1, AGRN,FOLR1
Skeletal and Muscular Disorders	5.01E-03-1.5E-02	BDNF,AGRN
Tumor Morphology	5.01E-03-1.99E-02	PMEL,BDNF,SFRP1
Hematopoiesis	7.51E-03-7.51E-03	AGRN
Infectious Disease	7.51E-03-2.24E-02	FOLR1
Reproductive System Development and Function	7.51E-03-7.51E-03	MMEL1
Visual System Development and Function	7.51E-03-7.51E-03	BDNF
Connective Tissue Development and Function	9.99E-03-9.99E-03	COL14A1
Gene Expression	9.99E-03-9.99E-03	NR3C2
Cell Signaling	1.06E-02-2.48E-02	AVPR2,VIPR1,BDNF,GPR26,AGRN
Post-Translational Modification	1.25E-02-1.25E-02	COL4A3
Protein Degradation	1.5E-02-1.5E-02	TIMP3
Protein Synthesis	1.5E-02-2.24E-02	TIMP3,FOLR1
Renal and Urological System Development and Function	1.5E-02-1.5E-02	TIMP3
Respiratory System Development and Function	1.5E-02-1.5E-02	SOD3
Vitamin and Mineral Metabolism	1.5E-02-2.73E-02	BDNF,FOLR1

Specific pre-hatching B responsive genes

Category	p-value	Genes
Endocrine System Development and Function	8.05E-07-4.03E-02	TTR,CYP19A1,TAC1,WFS1, SLCO1B1,MC4R,TCF7L2,LHX9
Molecular Transport	8.05E-07-4.13E-02	TTR,TAC1,MC4R,NPTX1,MC5R, GAD2,CYP19A1,RASGRP1,PENK,SLCO1B1, WFS1,TCF7L2,LHX9
Small Molecule Biochemistry	8.05E-07-4.99E-02	MC5R,GAD2,Gsta3,TTR,CYP19A1,TAC1,WFS1,SLCO1B1,MC4R,TCF7L2,LHX9
Behavior	2.39E-06-4.53E-02	MC5R,TTR,GAD2,CYP19A1, PENK,TAC1,NPTX1,MC4R
Carbohydrate Metabolism	2.44E-06-4.28E-02	GAD2,CYP19A1,TAC1,WFS1, SLCO1B1,MC4R,TCF7L2
Lipid Metabolism	4.89E-06-4.99E-02	MC5R,Gsta3,GAD2,TTR,CYP19A1, TAC1,SLCO1B1,MC4R,TCF7L2,LHX9
Nutritional Disease	9.88E-06-4.28E-02	MC5R,GAD2,CYP19A1,MC4R,TCF7L2
Psychological Disorders	2.27E-05-4.65E-02	TTR,GAD2,CYP19A1,PENK,SAG, TAC1,WFS1,MC4R,NPTX1,TCF7L2
Drug Metabolism	2.5E-05-4.03E-02	Gsta3,TTR,CYP19A1,TAC1,WFS1, SLCO1B1,MC4R,LHX9
Cell Death and Survival	3.28E-05-3.16E-02	GAD2,TTR,CYP19A1,RASGRP1,PENK,TAC1, EFEMP1,WFS1,NPTX1,AMIGO2,TCF7L2
Embryonic Development	7.03E-05-2.41E-02	CYP19A1,DLX5,TAC1,TCF7L2,LHX9
Organismal Development	7.03E-05-4.01E-02	MC5R,GAD2,CYP19A1,FBLN1,DLX5,TAC1, EFEMP1,MC4R,TCF7L2,LHX9
Cellular Function and Maintenance	7.19E-05-4.05E-02	Gsta3,CYP19A1,RASGRP1,DLX5, PENK,TAC1,EFEMP1,WFS1,MC4R,TCF7L2
Cell-To-Cell Signaling and Interaction	8.58E-05-4.77E-02	GAD2,CYP19A1,RASGRP1, PENK,TAC1,EFEMP1,NPTX1,MC4R
Reproductive System Development and Function	1.26E-04-4.53E-02	Gsta3,CYP19A1,TAC1,TRIM36, EFEMP1,MC4R,LHX9
Cell Signaling	1.29E-04-4.46E-02	MC5R,TTR,CYP19A1,TAC1,WFS1,MC4R
Nucleic Acid Metabolism	1.29E-04-3.16E-02	MC5R,TAC1,WFS1,SLCO1B1,MC4R
Cancer	1.42E-04-3.91E-02	Gsta3,TTR,CYP19A1,RASGRP1, FBLN1,EFEMP1,WFS1,SLCO1B1, NPTX1,TCF7L2
Endocrine System Disorders	1.42E-04-3.91E-02	TTR,GAD2,CYP19A1,WFS1,MC4R,TCF7L2
Reproductive System Disease	1.42E-04-3.91E-02	CYP19A1,EFEMP1,MC4R,TCF7L2,LHX9
Organismal Injury and Abnormalities	2.34E-04-4.77E-02	Gsta3,GAD2,CYP19A1,RASGRP1, PENK,TAC1,EFEMP1,NPTX1,MC4R
Nervous System Development and Function	2.65E-04-4.89E-02	TTR,GAD2,CYP19A1,DLX5,PENK, TAC1,NPTX1,MC4R,TCF7L2,AMIGO2
Cellular Compromise	3.06E-04-2.29E-02	TAC1,EFEMP1,WFS1,MC4R

Protein Synthesis	7.63E-04-4.03E-02	Gsta3,TTR,CYP19A1,RASGRP1,WFS1,MC4R
Hereditary Disorder	9.33E-04-4.16E-02	TTR,DLX5,TAC1,NPTX1,GAD2, CYP19A1,RASGRP1,PENK,FBLN1,SAG, EFEMP1,SLCO1B1,WFS1,TCF7L2
Neurological Disease	9.33E-04-4.65E-02	GAD2,TTR,CYP19A1,DLX5,PENK,SAG, TAC1,EFEMP1,WFS1,NPTX1,TCF7L2
Cardiovascular Disease	1.28E-03-4.03E-02	TTR,FBLN1
Cell Cycle	1.28E-03-1.53E-02	TAC1
Cell Morphology	1.28E-03-4.89E-02	GAD2,CYP19A1,DLX5,TAC1,NPTX1,TCF7L2
Cellular Assembly and Organization	1.28E-03-4.05E-02	GAD2,TTR,CYP19A1,DLX5,PENK,TAC1,NPTX1, MC4R
Cellular Development	1.28E-03-4.53E-02	TTR,CYP19A1,RASGRP1,DLX5,TAC1, TCF7L2, LHX9
Cellular Growth and Proliferation	1.28E-03-4.28E-02	GAD2,TTR,CYP19A1,RASGRP1,DLX5,PENK,FBLN1, TAC1,EFEMP1,TCF7L2,LHX9
Developmental Disorder	1.28E-03-4.65E-02	TTR,CYP19A1,DLX5,FBLN1,EFEMP1,SLCO1B1, MC4R,TCF7L2,LHX9
Digestive System Development and Function	1.28E-03-4.28E-02	GAD2,DLX5,TAC1,SLCO1B1,MC4R,TCF7L2
Energy Production	1.28E-03-3.16E-02	MC5R,CYP19A1,TAC1
Gastrointestinal Disease	1.28E-03-4.03E-02	GAD2,CYP19A1,DLX5,EFEMP1, SLCO1B1,WFS1,MC4R,TCF7L2
Immune Cell Trafficking	1.28E-03-3.74E-02	CYP19A1,RASGRP1,PENK,TAC1, NPTX1,MC4R
Metabolic Disease	1.28E-03-4.16E-02	GAD2,TTR,CYP19A1,EFEMP1, SLCO1B1,WFS1,MC4R,TCF7L2
Organ Development	1.28E-03-1.28E-02	CYP19A1,DLX5,TAC1,SLCO1B1,TCF7L2
Skeletal and Muscular Disorders	1.28E-03-4.29E-02	GAD2,CYP19A1,DLX5,FBLN1,PENK, SAG,TAC1,EFEMP1,TCF7L2
Tissue Development	1.28E-03-4.16E-02	TTR,CYP19A1,RASGRP1,DLX5, TAC1,EFEMP1,NPTX1,TCF7L2
Tissue Morphology	1.28E-03-4.01E-02	CYP19A1,RASGRP1,PENK,DLX5, FBLN1,TAC1,MC4R,TCF7L2
Tumor Morphology	1.28E-03-2.79E-02	CYP19A1,RASGRP1,TAC1,EFEMP1,TCF7L2
Organismal Functions	1.37E-03-4.79E-03	MC5R,Gsta3,CYP19A1,MC4R
Cardiovascular System Development and Function	2.57E-03-4.01E-02	CYP19A1,DLX5,FBLN1,TAC1
Immunological Disease	2.57E-03-1.78E-02	GAD2,CYP19A1,RASGRP1, TAC1,WFS1,TCF7L2
Post-Translational Modification	2.57E-03-2.57E-03	GAD2
Skeletal and Muscular System Development and Function	2.57E-03-1.53E-02	CYP19A1,DLX5,TAC1,EFEMP1
Free Radical Scavenging	3.85E-03-3.85E-03	Gsta3

Cellular Movement	5.13E-03-4.89E-02	CYP19A1,RASGRP1,DLX5,FBLN1, PENK,TAC1,NPTX1,MC4R
Hematological Disease	5.13E-03-1.78E-02	CYP19A1,FBLN1
Organ Morphology	5.13E-03-3.66E-02	DLX5,TCF7L2
Protein Trafficking	5.13E-03-8.96E-03	RASGRP1,TAC1
Cell-mediated Immune Response	5.5E-03-5.5E-03	RASGRP1,TAC1
Hypersensitivity Response	1.78E-02-4.28E-02	TAC1
DNA Replication, Recombination, and Repair	2.41E-02-2.41E-02	TAC1
Organismal Survival	2.66E-02-2.66E-02	TAC1

Interacting pre- and post-hatching B responsive genes: "cumulative effect"

Category	p-value	Genes
Neurological Disease	1.12E-05-4.93E-02	ATP7B,LRP2,TP73,GLRA3,MYO7A, TACR1,CA3,SLC6A11,OTOF,GAD1, ITGB4,KIAA1199,CACNA2D3
Molecular Transport	1.79E-04-4.34E-02	ATP7B,TP73,LRP2,SLC24A3,MYO7A,GRIP2, SLC4A11,TACR1,CA3,SLC6A11,OTOF, NPR3,KL,GAD1,PRDM16,CACNA2D3
Cancer	3.01E-04-4.34E-02	ATP7B,TP73,LRP2,SLC24A3,CDH6, TACR1,CA3,GDF10,ITGB4,KIAA1199, PRDM16,CACNA2D3,ALK
Lipid Metabolism	4.19E-04-3.59E-02	TACR1,SLC6A11,LRP2,KL,GAD1
Small Molecule Biochemistry	4.19E-04-4.34E-02	TACR1,SLC6A11,ATP7B,NPR3, SLC24A3,LRP2,KL,GAD1
Psychological Disorders	5.34E-04-4.61E-02	TACR1,SLC6A11,CA3,TP73,GAD1, CACNA2D3
Organismal Injury and Abnormalities	1.04E-03-4.34E-02	TACR1,CA3,ATP7B,NPR3,LRP2,TP73,KL,GAD1,ITGB4,CACNA2D3
Cell Signaling	1.53E-03-3.16E-02	TACR1,NPR3,TP73,ALK
Cell Cycle	1.53E-03-4.02E-02	TP73,KL,PRDM16
Cell Morphology	1.53E-03-4.78E-02	TACR1,OTOF,NPR3,KL,TP73, LRP2,MYO7A,GAD1,ITGB4
Cell-To-Cell Signaling and Interaction	1.53E-03-4.34E-02	TACR1,SLC6A11,OTOF,NPR3, LRP2,MYO7A,ITGB4,ALK
Cellular Function and Maintenance	1.53E-03-4.34E-02	TACR1,ATP7B,OTOF,TP73, LRP2,SLC24A3,KL,MYO7A,ITGB4
Developmental Disorder	1.53E-03-4.93E-02	ATP7B,TP73,KL,LRP2,SLC4A11, GRIP2,ITGB4,ZFHX4
Immunological Disease	1.53E-03-4.64E-02	CA3,LRP2,TP73,ALK
Nervous System Development and Function	1.53E-03-4.64E-02	TACR1,OTOF,LRP2,TP73,MYO7A, GAD1,ITGB4,CACNA2D3,ALK

Organ Development	1.53E-03-4.49E-02	NPR3,LRP2,TP73
Organ Morphology	1.53E-03-4.49E-02	NPR3,TP73,LRP2,ITGB4
Reproductive System Development and Function	1.53E-03-4.05E-02	TACR1,TP73,GAD1
Skeletal and Muscular Disorders	1.53E-03-4.64E-02	CA3,LRP2,TP73,KL,ZFHX4,CACNA2D3,ALK
Tissue Development	1.53E-03-4.49E-02	TACR1,NPR3,LRP2,TP73,ALK
Tissue Morphology	1.53E-03-4.64E-02	OTOF,NPR3,KL,TP73,LRP2, GAD1,ITGB4,PRDM16,ALK
Behavior	1.61E-03-4.78E-02	TACR1,TP73,GAD1,ALK
Cellular Assembly and Organization	3.06E-03-4.93E-02	OTOF,LRP2,TP73,MYO7A,ITGB4
Cellular Compromise	3.06E-03-4.93E-02	TACR1,TP73,ITGB4,PRDM16
Cellular Development	3.06E-03-4.2E-02	CA3,NPR3,TP73,MYO7A,ITGB4, PRDM16,ALK
Cellular Growth and Proliferation	3.06E-03-4.2E-02	TACR1,CA3,NPR3,TP73,ITGB4, PRDM16,ALK
Drug Metabolism	3.06E-03-2.12E-02	ATP7B,NPR3,LRP2,KL
Endocrine System Development and Function	3.06E-03-9.14E-03	TACR1,LRP2,GAD1
Organismal Development	3.06E-03-4.49E-02	TACR1,NPR3,TP73
Organismal Functions	3.06E-03-3.02E-02	TACR1
Post-Translational Modification	3.06E-03-5.67E-03	TP73,GAD1,ALK
Cellular Movement	3.29E-03-4.34E-02	TACR1,OTOF,GAD1,ITGB4,ALK
Gene Expression	4.01E-03-2.27E-02	TP73,ALK
Gastrointestinal Disease	4.27E-03-4.05E-02	TACR1,CA3,ATP7B,TP73,GAD1, CDH6,ITGB4,KIAA1199,CACNA2D3
Cell Death and Survival	4.58E-03-4.93E-02	TACR1,ATP7B,CA3,LRP2,TP73,KL,ALK
Free Radical Scavenging	4.58E-03-1.79E-02	CA3,KL,PRDM16
Metabolic Disease	4.58E-03-4.93E-02	CA3,ATP7B,NPR3,KL,TP73,LRP2
Hematological Disease	6.1E-03-4.49E-02	ATP7B,TP73,KL,GAD1,ALK
Skeletal and Muscular System Development and Function	7.62E-03-4.64E-02	NPR3,KL
Cardiovascular Disease	9.14E-03-4.78E-02	CA3,NPR3,KL,CACNA2D3
Tumor Morphology	9.14E-03-4.34E-02	ATP7B,TP73,ITGB4
Cardiovascular System Development and Function	9.79E-03-1.67E-02	TACR1,NPR3,TP73,KL
Immune Cell Trafficking	1.07E-02-1.07E-02	TACR1

Endocrine System Disorders	1.57E-02-4.78E-02	TACR1,CA3,TP73,KL,LRP2,ITGB4, CACNA2D3
Reproductive System Disease	1.84E-02-3.45E-02	TACR1,SLC24A3,TP73,LRP2,ITGB4,ALK
Protein Synthesis	2.12E-02-4.64E-02	NPR3,TP73
Organismal Survival	2.32E-02-2.32E-02	ATP7B,TP73,KL,ALK
Hypersensitivity Response	2.72E-02-2.72E-02	TACR1
Embryonic Development	3.61E-02-4.49E-02	TACR1,NPR3

(b) Hypothalamus

Pre- and post-hatching B responsive genes

Category	*p-value*	Genes
Behavior	6.73E-04-4.16E-02	RACGAP1,CCK
Cancer	6.73E-04-4.22E-02	CALB2,RACGAP1,PROX1,CCK
Carbohydrate Metabolism	6.73E-04-3.84E-02	KCNK5,CCK
Cardiovascular System Development and Function	6.73E-04-4.35E-02	PROX1
Cell Morphology	6.73E-04-4.35E-02	NTNG1,PROX1,CCK
Cell-To-Cell Signaling and Interaction	6.73E-04-4.8E-02	CCK
Digestive System Development and Function	6.73E-04-2.4E-02	CCK
Drug Metabolism	6.73E-04-2.02E-03	CCK
Embryonic Development	6.73E-04-9.38E-03	PROX1
Endocrine System Development and Function	6.73E-04-1.35E-03	CCK
Hereditary Disorder	6.73E-04-6.73E-04	RD3
Immunological Disease	6.73E-04-6.73E-04	PROX1
Nervous System Development and Function	6.73E-04-3.64E-02	NTNG1,CALB2,PROX1,CCK
Nucleic Acid Metabolism	6.73E-04-2.73E-02	ENTPD2,CCK
Organ Development	6.73E-04-9.38E-03	PROX1
Organ Morphology	6.73E-04-6.73E-04	CCK
Organismal Development	6.73E-04-4.35E-02	PROX1

Organismal Functions	6.73E-04-6.73E-04	CCK
Skeletal and Muscular System Development and Function	6.73E-04-6.73E-04	CCK
Small Molecule Biochemistry	6.73E-04-4.8E-02	ENTPD2,PROX1,CCK
Tissue Development	6.73E-04-3.96E-02	NTNG1,PROX1,CCK
Cell Cycle	1.35E-03-1.35E-03	CCK
Cellular Growth and Proliferation	1.35E-03-2.69E-03	CCK
Developmental Disorder	1.35E-03-1.35E-03	CCK
Endocrine System Disorders	1.35E-03-3.71E-02	CALB2,RACGAP1,CCK
Gastrointestinal Disease	1.35E-03-4.03E-02	CCK
Cellular Movement	2.02E-03-3.96E-02	PROX1
DNA Replication, Recombination, and Repair	2.02E-03-2.73E-02	ENTPD2,RACGAP1
Immune Cell Trafficking	2.02E-03-2.02E-03	PROX1
Molecular Transport	2.02E-03-4.95E-02	KCNK5,RACGAP1,CBLN4,CCK
Cellular Development	2.69E-03-4.35E-02	NTNG1,PROX1,CCK
Energy Production	2.69E-03-2.73E-02	ENTPD2
Reproductive System Disease	3.34E-03-1.67E-02	CALB2,RACGAP1
Cell Death and Survival	4.7E-03-4.93E-02	CALB2,CCK
Cell Signaling	6.71E-03-4.93E-02	PROX1,CCK
Cellular Assembly and Organization	7.18E-03-3.44E-02	NTNG1,CCK
Cellular Function and Maintenance	7.18E-03-1.87E-02	NTNG1,CCK
Nutritional Disease	8.71E-03-2.26E-02	PROX1,CCK
Psychological Disorders	8.71E-03-2.26E-02	CCK
Lipid Metabolism	1.54E-02-1.54E-02	PROX1
Post-Translational Modification	1.6E-02-1.6E-02	CCK
Reproductive System Development and Function	1.8E-02-1.8E-02	FGL2
Metabolic Disease	3.71E-02-3.71E-02	CCK

Dermatological Diseases and Conditions	4.03E-02-4.03E-02	PROX1
Amino Acid Metabolism	4.8E-02-4.8E-02	CCK

Specific pre-hatching B responsive genes

Category	*p-value*	Genes
Lipid Metabolism	4.76E-06-2.32E-02	LHCGR,TRPC4,CGA,HPGDS,PRKCB
Molecular Transport	4.76E-06-4.25E-02	CA3,ITPR3,LHCGR,TRPC4,CGA, HPGDS,PRDM16,RASD1,PRKCB
Small Molecule Biochemistry	4.76E-06-4.74E-02	LHCGR,TRPC4,CGA,HPGDS,RASD1,PRKCB
Drug Metabolism	3.25E-05-3.96E-02	LHCGR,CGA,PRKCB
Endocrine System Development and Function	3.25E-05-3.89E-02	ITPR3,LHCGR,CGA
Cell Signaling	5.91E-05-4.96E-02	ITPR3,TPD52L1,LHCGR,TRPC4, CGA,RASD1,PRKCB
Vitamin and Mineral Metabolism	5.91E-05-4.25E-02	ITPR3,LHCGR,TRPC4,CGA,PRKCB
Protein Synthesis	2.42E-04-9.5E-03	LHCGR,CGA
Cancer	3.24E-04-4.04E-02	CA3,ITPR3,TPD52L1,LHCGR,HPGDS,IRX2, CGA,PRDM16,PRKCB
Amino Acid Metabolism	4.39E-04-3.54E-02	TRPC4,CGA,PRKCB
Cardiovascular Disease	5.93E-04-4.88E-02	CA3,LHCGR,TRPC4,CGA,HPGDS,PRKCB
Cell Cycle	7.34E-04-4.18E-02	TPD52L1,CGA,PRDM16,PRKCB
Developmental Disorder	7.34E-04-1.46E-02	LHCGR,CGA
Endocrine System Disorders	7.34E-04-2.32E-02	CA3,ITPR3,LHCGR,CGA,PRKCB
Hereditary Disorder	7.34E-04-4.74E-02	CA3,LHCGR,CGA
Metabolic Disease	7.34E-04-4.88E-02	CA3,ITPR3,LHCGR,PRKCB
Neurological Disease	7.34E-04-4.88E-02	CA3,HPGDS,CGA,PRKCB
Organismal Injury and Abnormalities	7.34E-04-2.47E-02	CA3,CGA,HPGDS,PRKCB
Reproductive System Development and Function	7.34E-04-4.39E-02	LHCGR,CGA,PRKCB
Reproductive System Disease	7.34E-04-2.83E-02	LHCGR,CGA
Tissue Morphology	7.34E-04-4.04E-02	LHCGR,HPGDS,CGA,PRDM16,PRKCB

Tumor Morphology	7.34E-04-9.5E-03	LHCGR,IRX2
Organ Morphology	1.47E-03-7.32E-03	ITPR3,CGA
Cell Death and Survival	1.83E-03-3.4E-02	CA3,ITPR3,LHCGR,TPD52L1, HPGDS,PRDM16,PRKCB
Cell Morphology	2.2E-03-4.32E-02	ITPR3,LHCGR,TRPC4
Cellular Compromise	2.2E-03-1.39E-02	TRPC4,HPGDS,PRDM16
Cellular Development	2.2E-03-4.32E-02	LHCGR,TRPC4,CGA,PRDM16,PRKCB
Embryonic Development	2.2E-03-3.57E-02	LHCGR,CGA,PRKCB
Organ Development	2.2E-03-3.57E-02	LHCGR,CGA
Organismal Development	2.2E-03-4.32E-02	ITPR3,LHCGR,TRPC4,CGA
Tissue Development	2.2E-03-4.32E-02	LHCGR,TRPC4,HPGDS,CGA
Cell-To-Cell Signaling and Interaction	2.93E-03-3.96E-02	LHCGR,PRKCB
Cellular Assembly and Organization	2.93E-03-4.53E-02	ITPR3,RASD1
Cellular Function and Maintenance	2.93E-03-2.32E-02	ITPR3,LHCGR,PRKCB
Cellular Movement	2.93E-03-4.74E-02	CA3,CGA,IRX2,PRKCB
Nervous System Development and Function	2.93E-03-9.5E-03	LHCGR,CGA,HPGDS,RASD1
Free Radical Scavenging	4.23E-03-4.23E-03	CA3,PRDM16
Behavior	4.4E-03-4.44E-02	ITPR3,LHCGR,HPGDS, RASD1,PRKCB
Cardiovascular System Development and Function	4.4E-03-4.32E-02	TRPC4
Cellular Growth and Proliferation	4.4E-03-3.89E-02	CA3,LHCGR,TRPC4,CGA,HPGDS, PRDM16,RASD1,PRKCB
Renal and Urological System Development and Function	4.4E-03-3.75E-02	LHCGR,CGA
Nucleic Acid Metabolism	4.42E-03-2.18E-02	LHCGR,CGA,RASD1,PRKCB
Carbohydrate Metabolism	5.13E-03-4.74E-02	LHCGR,PRKCB
Gastrointestinal	5.86E-03-4.6E-02	CA3,ITPR3,PRKCB

Disease		
Infectious Disease	5.86E-03-1.17E-02	CA3,PRKCB
Digestive System Development and Function	7.32E-03-1.46E-02	ITPR3
Energy Production	1.1E-02-1.1E-02	PRKCB
Respiratory Disease	1.1E-02-1.1E-02	CA3
Organismal Functions	1.17E-02-2.75E-02	HPGDS,PRKCB
Nutritional Disease	1.24E-02-4.43E-02	CA3,PRKCB
Psychological Disorders	1.24E-02-4.18E-02	CA3,PRKCB
Skeletal and Muscular Disorders	1.39E-02-2.25E-02	CA3,PRKCB
Post-Translational Modification	1.6E-02-1.82E-02	PRKCB
Renal and Urological Disease	1.6E-02-2.32E-02	CA3
Immunological Disease	2.05E-02-2.61E-02	CA3,ITPR3,HPGDS,PRKCB
DNA Replication, Recombination, and Repair	3.4E-02-3.4E-02	TPD52L1
Skeletal and Muscular System Development and	3.61E-02-3.61E-02	HPGDS
Immune Cell Trafficking	4.74E-02-4.74E-02	IRX2,PRKCB

Specific post-hatching B responsive genes

Category	p-value	Genes
Neurological Disease	1.77E-07-4.82E-02	CNKSR2,GABRA5,GRIN2A,RASD2,TYRO3,HTR1D, COL4A2,RGS12,CNJ4,SATB2,HTR2C,SSTR5, PCDH8,ADORA1,GABRD,NGEF,CYP27A1,ABCA4, SEMA5A,DCLK3,EPHA3,RASL12,BHLHE40, ARHGEF6,HTR3A,ALDH1A2,ARPP21
Psychological Disorders	1.77E-07-2.58E-02	NGEF,GABRA5,GRIN2A,CNKSR2,RASD2,CYP27A1, SEMA5A,HTR1D,COL4A2,DCLK3,RGS12, RASL12,HTR2C,SATB2,KCNJ4,BHLHE40, SSTR5,HTR3A,ARPP21,PCDH8,GABRD,ADORA1
Nervous System Development and Function	6.12E-06-4.46E-02	GRIN2A,GABRA5,CRHR2,RASD2,TYRO3,HTR1D,SA TB2,HTR2C,FEZF2,PCDH8,ADORA1,GABRD,EURO D6,LMO7,ABCA4,SEMA5A,EMX1,CRTAC1,EPHA3, BTB18,BHLHE40,ALDH1A2,HTR3A,MAFB,ARPP21
Organ Morphology	6.12E-06-4.46E-02	ZBTB18,NEUROD6,ABCA4,ALDH1A2, TYRO3,FEZF2,EMX1

Cell-To-Cell Signaling and Interaction	8.68E-06-4.09E-02	GABRA5,CRHR2,GRIN2A,RASD2,NTN4,ANTXR1, TYRO3,HTR1D,HTR2C,HTR3A,PCDH8,ADORA1, GABRD
Hereditary Disorder	2.05E-05-2.25E-02	NGEF,GABRA5,GRIN2A,RASD2,COL9A1,CYP27A1, ABCA4,SEMA5A,HTR1D,BUB1B,RGS12,RASL12, HTR2C,KCNJ4,BHLHE40,SSTR5,HTR3A,ARHGEF6, CISH,ARPP21,PCDH8,GABRD
Skeletal and Muscular Disorders	3.16E-05-4.31E-02	RASD2,GABRA5,NGEF,GRIN2A,COL9A1,SEMA5A, ANTXR1,HTR1D,RASL12,HTR2C,KCNJ4,SATB2, BHLHE40,HTR3A,MAFB,ARPP21,ADORA1,GABRD
Nutritional Disease	3.98E-05-2.25E-02	CACNG3,HTR2C,CRHR2,GRIN2A,GABRA5, HTR3A,SSTR5,HTR1D,ADORA1,GABRD
Organismal Injury and Abnormalities	7.7E-05-2.97E-02	HTR2C,GRIN2A,GABRA5,COL9A1,HTR3A, TYRO3,HTR1D,COL4A2,ADORA1,GABRD
Cell Signaling	9.93E-05-2.63E-02	HTR2C,CRHR2,CISH,SSTR5, DGKG,HTR1D,ADORA1,RGS12
Post-Translational Modification	9.93E-05-1.68E-02	CRHR2,CISH,DGKG,ADORA1
Cellular Assembly and Organization	1.27E-04-4.71E-02	NEUROD6,SEMA5A,FEZF2,ANTXR1,TYRO3, MRAS,CRTAC1,FILIP1L,FMNL1,PCDH8, EPHA3,BUB1B
Behavior	1.34E-04-4.46E-02	HTR2C,GRIN2A,RASD2,GABRA5,CRHR2, HOMER2,BHLHE40,FEZF2,PCDH8, GABRD,ADORA1
Cellular Movement	2.94E-04-4.09E-02	SATB2,GABRA5,SEMA5A,FMNL1,MAFB, EPHA3,ADORA1
Developmental Disorder	3.12E-04-4.46E-02	GABRA5,GRIN2A,COL9A1,CYP27A1, BUB1B,HTR2C,SATB2,ARHGEF6,SSTR5,HTR3A, ALDH1A2,RSPO2,GABRD,ADORA1
Tissue Development	9.11E-04-4.46E-02	NEUROD6,GRIN2A,GABRA5,NTN4, ABCA4,SEMA5A,TYRO3,EMX1,ANTXR1, FILIP1L,ZBTB18,BHLHE40,ALDH1A2,FEZF2, PCDH8,MAFB,ADORA1
Gastrointestinal Disease	1.44E-03-4.46E-02	HTR2C,SATB2,GRIN2A,COL9A1,SSTR5, ANTXR1,HTR3A,RSPO2,COL4A2
Embryonic Development	1.49E-03-4.46E-02	NEUROD6,NTN4,ABCA4,ANTXR1,TYRO3,EMX1, FILIP1L,COL4A2,EPHA3,ZBTB18,SATB2,FEZF2, ALDH1A2,RSPO2,MAFB
Organ Development	1.49E-03-4.46E-02	ZBTB18,NEUROD6,ABCA4,FEZF2,EMX1,ALDH1A2, TYRO3,FILIP1L,MAFB
Organismal Development	1.49E-03-4.46E-02	NEUROD6,CRHR2,ABCA4,ANTXR1,TYRO3,EMX1, COL4A2,FILIP1L,ZBTB18,SATB2,ALDH1A2, FEZF2,RSPO2,MAFB
Digestive System Development and Function	1.81E-03-4.46E-02	SATB2,HTR2C,CRHR2,HOMER2,ANTXR1, FEZF2,RSPO2,GABRD
Tissue Morphology	1.92E-03-4.09E-02	GRIN2A,GABRA5,COL9A1,SEMA5A,ANTXR1, CRTAC1,EPHA3,COL4A2,ZBTB18,SATB2, ALDH1A2,RSPO2,MAFB,ADORA1

Cardiovascular Disease	2.57E-03-4.6E-02	GRIN2A,GABRA5,TYRO3,ANTXR1,SSTR5, EPHA3,ADORA1,GABRD,RASL12
Inflammatory Disease	3.11E-03-1.88E-02	HTR2C,GRIN2A,COL9A1,HTR3A,ADORA1
Skeletal and Muscular System Development and Function	3.7E-03-4.46E-02	COL9A1,BHLHE40,ALDH1A2,RSPO2, FILIP1L,MAFB
Carbohydrate Metabolism	3.79E-03-3.73E-02	HTR2C,ABCA4,MRAS,ADORA1
Cell Cycle	3.79E-03-3.36E-02	BHLHE40,SSTR5,FMNL1,BUB1B
Cell Morphology	3.79E-03-4.93E-02	GRIN2A,CRHR2,GABRA5,NGEF,NTN4,SEMA5A, ANTXR1,COL4A2,FMNL1,BHLHE40,RSPO2,PCDH8, ADORA1
Cellular Function and Maintenance	3.79E-03-4.93E-02	NEUROD6,GRIN2A,CRHR2,GABRA5, NTN4,ANTXR1,TYRO3,EPHA3,COL4A2, ZBTB18,FEZF2,MRAS,PCDH8,MAFB,ADORA1
Cellular Growth and Proliferation	3.79E-03-4.09E-02	MRAS,COL4A2,MAFB
Lipid Metabolism	3.79E-03-4.82E-02	HTR2C,HOMER2,CYP27A1,ABCA4,BHLHE40, ALDH1A2,MRAS,ADORA1,BUB1B
Molecular Transport	3.79E-03-4.82E-02	CRHR2,GRIN2A,GABRA5,CYP27A1,KCNS1,ABCA4, RGS12,HTR2C,KCNJ4,BHLHE40,SATB1,MRAS, ADORA1
Small Molecule Biochemistry	3.79E-03-4.82E-02	HTR2C,GRIN2A,CRHR2,HOMER2,CYP27A1, ABCA4,BHLHE40,ALDH1A2,MRAS,RGS12,BUB1B, ADORA1
Visual System Development	3.79E-03-1.88E-02	ABCA4
Cell Death and Survival	4.67E-03-4.46E-02	GABRA5,CRHR2,NTN4,ALDH1A2,ANTXR1, COL4A2,GABRD
Cancer	5.52E-03-4.72E-02	GRIN2A,LMO7,ABCA4,SEMA5A,ANTXR1,EPHA3, COL4A2,RGS12,BUB1B,RASL12,CACNG3,SATB2, BHLHE40,SATB1,SSTR5,ALDH1A2,HTR3A,MAFB
Reproductive System Disease	5.52E-03-4.82E-02	CACNG3,SATB2,HTR2C,ABCA4,BHLHE40, ALDH1A2,SSTR5,ANTXR1,RASL12
Endocrine System Disorders	5.75E-03-4.82E-02	HTR2C,ABCA4,ALDH1A2,SSTR5
Metabolic Disease	5.75E-03-1.13E-02	HTR2C,CYP27A1,SSTR5
Cellular Development	7.57E-03-4.46E-02	ZBTB18,NEUROD6,GABRA5,BHLHE40,SEMA5A, ALDH1A2,EMX1,RSPO2,MRAS,ARPP21,COL4A2, MAFB
DNA Replication, Recombination, and Repair	7.57E-03-7.57E-03	ABCA4
Drug Metabolism	7.57E-03-4.09E-02	HTR2C,HOMER2,ALDH1A2,ADORA1
Immune Cell Trafficking	7.57E-03-7.57E-03	ADORA1

Inflammatory Response	7.57E-03-3.73E-02	TYRO3,MRAS,ADORA1
Nucleic Acid Metabolism	7.57E-03-4.09E-02	HTR2C,CRHR2,ABCA4,RGS12,ADORA1
Tumor Morphology	7.57E-03-7.57E-03	SEMA5A
Cellular Compromise	1.13E-02-3.36E-02	NGEF,GABRA5,SEMA5A,FMNL1,ADORA1,GABRD
Energy Production	1.13E-02-2.63E-02	CYP27A1,ALDH1A2
Organismal Functions	1.13E-02-4.76E-02	CRHR2,GRIN2A,RASD2,FEZF2
Amino Acid Metabolism	1.16E-02-4.49E-02	HTR2C,GRIN2A,HOMER2,ADORA1
Immunological Disease	1.51E-02-1.51E-02	HTR3A
Reproductive System Development	1.51E-02-4.09E-02	SSTR5,GABRD
Lymphoid Tissue Structure	1.88E-02-4.46E-02	ALDH1A2,MAFB
Protein Synthesis	1.88E-02-1.88E-02	SATB1
Protein Trafficking	1.88E-02-1.88E-02	SATB1
Endocrine System Development	2.25E-02-4.82E-02	HTR2C,HOMER2
Hepatic System Disease	2.25E-02-3.36E-02	SSTR5
Cardiovascular System Development	3.62E-02-3.62E-02	CRHR2,COL4A2

Table A9. Gene lists of the common significantly down-regulated genes (FDR ≤ 0.20) across the pair-wise comparison between the RNA-seq and Microarrays data obtained from the RankProducts statistics. FC indicates the fold change. Genes are sorted using the fold changes from RNA-seq.

(a) Contrast BC vs CC (2nd class vs 1st class): down-regulated genes in 2nd class

Ensembl ID	Description	RNA-seq: FC	Microarrays: FC
ENSGALG00000015143	transthyretin	-18.1960	-3.9350
ENSGALG00000012908	solute carrier family 13 (sodium/sulfate symporters), member 4	-3.6478	-15.2708
ENSGALG00000011369	LIM homeobox 8	-3.6024	-3.2358
ENSGALG00000011859	eye-globin	-3.5947	-6.9906
ENSGALG00000016553	transmembrane protein 27	-3.2546	-2.5394
ENSGALG00000018557	superoxide dismutase 3, extracellular	-2.8748	-3.2401
ENSGALG00000005628	collagen, type IX, alpha 3	-2.7318	-3.3814
ENSGALG00000015918	EF-hand calcium binding protein 1	-2.3182	-2.9294
ENSGALG00000014884	ISL LIM homeobox 1	-2.2846	-6.3441
ENSGALG00000013168	islet amyloid polypeptide	-2.2804	-3.4750
ENSGALG00000013362	calcium binding protein 7	-2.2529	-4.1170
ENSGALG00000012911	synaptotagmin X	-2.1913	-3.5970
ENSGALG00000014117	arginine vasopressin (neurophysin II, diuretic hormone, diabetes insipidus, neurohy)	-2.1489	-3.0358
ENSGALG00000009471	phosphatidic acid phosphatase type 2 domain containing 1A	-2.1392	-2.6570
ENSGALG00000004729	solute carrier family 7, (neutral amino acid transporter, y+ system) member 10	-2.1070	-3.3570
ENSGALG00000002161	similar to MGC80370 protein	-2.0896	-2.1696
ENSGALG00000012381	neurexophilin 2	-2.0355	-3.0618
ENSGALG00000007772	cerebellin 4 precursor	-2.0005	-3.1788
ENSGALG00000016428	ectonucleotide	-1.9213	-2.6038

ENSGALG00000015890	hypothetical LOC421856	-1.8966	-9.2777
ENSGALG00000007945	crystallin, alpha B	-1.8723	-2.1622
ENSGALG00000009515	guanine nucleotide binding protein (G protein), gamma 11	-1.8053	-2.6799
ENSGALG00000015023	serine/threonine kinase 32B	-1.7229	-2.8817
ENSGALG00000002652	frizzled homolog 10 (Drosophila)	-1.7185	-3.1692
ENSGALG00000014233	fibulin 1	-1.6910	-3.4437
ENSGALG00000013775	cadherin 19, type 2	-1.6873	-3.7677
ENSGALG00000005030	dedicator of cytokinesis 10	-1.6581	-2.8015
ENSGALG00000012362	thrombospondin, type I, domain containing 7B	-1.5833	-2.2458
ENSGALG00000009424	forkhead box P2	-1.5490	-3.8299
ENSGALG00000015744	tumor protein D52	-1.5429	-2.1328
ENSGALG00000005293	guanine nucleotide binding protein (G protein), gamma 13	-1.5404	-2.5342
ENSGALG00000016396	collectin sub-family member 11	-1.5396	-2.2619
ENSGALG00000016324	glutathione S-transferase alpha 3	-1.4809	-3.3354
ENSGALG00000013615	mitochondrial tumor suppressor 1	-1.4476	-2.2854

(b) BC vs CC (2^{nd} class vs 1^{st} class): up-regulated genes in 2^{nd} class

Ensembl ID	Description	RNA-seq: FC	Microarrays: FC
ENSGALG00000006676	retinaldehyde binding protein 1	1.9513	2.3279
ENSGALG00000012327	inhibin, beta A	1.8766	2.5241
ENSGALG00000000184	solute carrier family 27 (fatty acid transporter), member 6	1.8676	2.5283
ENSGALG00000015720	chondrolectin	1.7736	2.4228
ENSGALG00000009705	ryanodine receptor 3	1.4683	2.1439

(c) CB vs CC (2nd class vs 1st class): down-regulated genes in 2nd class

Ensembl ID	Description	RNA-seq: FC	Microarrays: FC
ENSGALG00000012908	solute carrier family 13	-2.8656	-4.1753
ENSGALG00000011859	eye-globin	-2.6732	-4.1945
ENSGALG00000016553	transmembrane protein 27	-2.2201	-2.6108
ENSGALG00000002652	frizzled homolog 10 (Drosophila)	-1.5761	-3.6016

(d) CB vs CC (2nd class vs 1st class): up-regulated genes in 2nd class

Ensembl ID	Description	RNA-seq: FC	Microarrays: FC
ENSGALG00000002577	StAR-related lipid transfer (START)	1.9365	2.2739

(e) BB vs CC (2nd class vs 1st class): down-regulated genes in 2nd class

Ensembl ID	Description	RNA-seq: FC	Microarrays: FC
ENSGALG00000015143	transthyretin	-28.0977	-5.3525
ENSGALG00000011859	eye-globin	-4.5587	-12.6967
ENSGALG00000012908	solute carrier family 13 (sodium/sulfate symporters), member 4	-3.7024	-26.6424
ENSGALG00000016553	transmembrane protein 27	-3.5302	-2.8991
ENSGALG00000011369	LIM homeobox 8	-3.2484	-3.1794
ENSGALG00000018557	superoxide dismutase 3, extracellular	-2.8380	-3.7259
ENSGALG00000005628	collagen, type IX, alpha 3	-2.7236	-4.0469
ENSGALG00000014967	synaptic vesicle glycoprotein 2C	-2.4284	-2.1571
ENSGALG00000013362	calcium binding protein 7	-2.3472	-4.1754
ENSGALG00000014884	ISL LIM homeobox 1	-2.1360	-6.6107
ENSGALG00000013168	islet amyloid polypeptide	-2.1270	-3.4644
ENSGALG00000007772	cerebellin 4 precursor	-2.1090	-3.1000
ENSGALG00000016428	ectonucleotide	-2.0265	-2.3026

ENSGALG00000015673	zinc finger homeodomain 4	-1.9412	-2.2448
ENSGALG00000009006	six transmembrane epithelial antigen of the prostate 1	-1.8268	-2.2244
ENSGALG00000015890	hypothetical LOC421856	-1.8257	-8.0570
ENSGALG00000014233	fibulin 1	-1.6756	-2.9153
ENSGALG00000009424	forkhead box P2	-1.6691	-3.4193
ENSGALG00000009471	phosphatidic acid phosphatase type 2 domain containing 1A	-1.6659	-2.1419
ENSGALG00000009515	guanine nucleotide binding protein (G protein), gamma 11	-1.6160	-3.0470
ENSGALG00000015685	hypothetical gene supported by	-1.6069	-2.3245
ENSGALG00000016324	glutathione S-transferase alpha 3	-1.5903	-2.4798
ENSGALG00000013616	similar to opioid receptor B	-1.4556	-2.2046

(f) BB vs CC (2^{nd} class vs 1^{st} class): up-regulated genes in 2^{nd} class

Ensembl ID	Description	RNA-seq: FC	Microarrays: FC
ENSGALG00000015018	calsequestrin 2 (cardiac muscle)	3.1243	3.9900
ENSGALG00000001695	gamma-aminobutyric acid (GABA) A receptor, alpha 6	2.6068	3.3897
ENSGALG00000012120	engrailed homeobox 1	2.0521	4.3129
ENSGALG00000004527	unc-13 homolog C (C. elegans)	2.0334	3.1369

(g) CB vs BC (2^{nd} class vs 1^{st} class): up-regulated genes in 2^{nd} class

Ensembl ID	Description	RNA-seq: FC	Microarrays: FC
ENSGALG00000014117	arginine vasopressin (neurophysin II, antidiuretic hormone, diabetes	4.2093	5.7604
ENSGALG00000021552	RAP2B, member of RAS oncogene family	1.4544	2.0854

(h) BB vs BC (2nd class vs 1st class): down-regulated genes in 2nd class

Ensembl ID	Description	RNA-seq: FC	Microarrays: FC
ENSGALG00000000184	solute carrier family 27 (fatty acid transporter), member 6	-1.6835	-2.6016
ENSGALG00000016244	leucine rich repeat containing 6	-1.5007	-4.0329
ENSGALG00000008058	p21 (CDKN1A)-activated kinase 3	-1.4974	-3.7297
ENSGALG00000006445	aryl-hydrocarbon receptor nuclear	-1.4794	-2.4832

(i) BB vs BC (2nd class vs 1st class): up-regulated genes in 2nd class

Ensembl ID	Description	RNA-seq: FC	Microarrays: FC
ENSGALG00000004527	unc-13 homolog C (C. elegans)	3.0010	4.1285
ENSGALG00000006811	Zic family member 1 (odd-paired homolog, Drosophila)	2.9334	2.1557
ENSGALG00000001695	gamma-aminobutyric acid (GABA) A receptor, alpha 6	2.4376	3.8743
ENSGALG00000015018	calsequestrin 2 (cardiac muscle)	2.4365	2.8243
ENSGALG00000012120	engrailed homeobox 1	2.1794	4.9536
ENSGALG00000008881	regulator of G-protein signalling 3	2.1723	3.3568
ENSGALG00000012362	thrombospondin, type I, domain containing 7B	2.0578	2.4080
ENSGALG00000001282	gamma-aminobutyric acid (GABA) A receptor, delta	2.0039	3.0420
ENSGALG00000000681	p21 protein	1.9490	2.8240
ENSGALG00000002652	frizzled homolog 10 (Drosophila)	1.8741	4.2778
ENSGALG00000015472	chromodomain helicase DNA binding protein 7	1.8383	4.3689
ENSGALG00000013615	mitochondrial tumor suppressor 1	1.8085	4.6785
ENSGALG00000024428	chromosome 17 open reading frame 67	1.6762	2.6410

(l) BB vs BC (2nd class vs 1st class): down-regulated genes in 2nd class

Ensembl ID	Description	RNA-seq: FC	Microarrays: FC
ENSGALG00000015143	transthyretin	-6.7550	-2.8937

(m) BB vs BC (2^{nd} class vs 1^{st} class): up-regulated genes in 2^{nd} class

Ensembl ID	Description	RNA-seq: FC	Microarrays: FC
ENSGALG00000000681	p21 protein (Cdc42/Rac)-activated kinase	1.7877	2.7422
ENSGALG00000001695	gamma-aminobutyric acid (GABA) A receptor, alpha 6	3.6483	3.9011
ENSGALG00000003149	inositol 1,4,5-triphosphate receptor, type 3	2.9759	2.3960
ENSGALG00000004527	unc-13 homolog C (C. elegans)	4.4623	4.2945
ENSGALG00000006811	Zic family member 1 (odd-paired homolog, Drosophila)	3.4901	2.8128
ENSGALG00000008881	regulator of G-protein signalling 3	2.3773	3.7291
ENSGALG00000008908	neurogenic differentiation 1	2.5441	2.4565
ENSGALG00000010939	lin-7 homolog A (*C. elegans*)	2.5249	3.1744
ENSGALG00000012120	engrailed homeobox 1	2.6720	6.5281
ENSGALG00000012522	parvalbumin	2.2450	2.2449
ENSGALG00000015018	calsequestrin 2 (cardiac muscle)	3.2214	5.4047
ENSGALG00000015472	chromodomain helicase DNA binding protein 7	2.1577	4.6159
ENSGALG00000023818	heat shock protein 25	1.8641	4.3193

Table A10. Hippocampal genes that were consistently down-regulated in the adult birds that were exposed to B *in ovo* compared to the control birds (BC *vs* CC and BB *vs* CC contrasts) in both RNA-seq and Microarrays. Genes are sorted by RNA-seq fold changes (FC).

Ensembl ID	Description
ENSGALG00000015143	transthyretin
ENSGALG00000012908	solute carrier family 13 (sodium/sulfate symporters), member 4
ENSGALG00000011369	LIM homeobox 8
ENSGALG00000011859	eye-globin
ENSGALG00000016553	transmembrane protein 27
ENSGALG00000018557	superoxide dismutase 3, extracellular
ENSGALG00000005628	collagen, type IX, alpha 3
ENSGALG00000014884	ISL LIM homeobox 1
ENSGALG00000013168	islet amyloid polypeptide
ENSGALG00000013362	calcium binding protein 7
ENSGALG00000009471	phosphatidic acid phosphatase type 2 domain containing 1A
ENSGALG00000007772	cerebellin 4 precursor
ENSGALG00000016428	ectonucleotide pyrophosphatase/phosphodiesterase 2
ENSGALG00000015890	hypothetical LOC421856
ENSGALG00000009515	guanine nucleotide binding protein (G protein), gamma 11
ENSGALG00000014233	fibulin 1
ENSGALG00000009424	forkhead box P2
ENSGALG00000016324	glutathione S-transferase alpha 3