EFFECTS OF MATERNAL STRESS AND CORTISOL TREATMENT ON OFFSPRING ANXIETY BEHAVIOUR AND STRESS RESPONSES IN ZEBRAFISH (*Danio rerio*) AND LARGEMOUTH BASS (*Micropterus salmoides*)

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ABSTRACT

In fish, maternal stress prior to spawn has been reported to have effects on offspring phenotype. Cortisol, the main glucocorticoid (GC) stress hormone, has been proposed as a potential mediator of such effects because of its organizational role in early teleost development. The present thesis tested whether maternal social stress or treatment with cortisol (as a proxy for maternal stress) prior to spawn affects the cortisol response to stress and anxiety-related behaviours in offspring. In zebrafish (*Danio rerio*), offspring of dominant females exhibited greater boldness at 6 days post-fertilization (DPF). Interestingly, offspring of females that engaged in social interactions, regardless of the resulting social status of the two females, exhibited greater survival at 1 DPF, a greater fear-related decrease in activity in response to bright light at 6 DPF, and decreased baseline whole-body cortisol content at 0 and 30 DPF. A field experiment with wild largemouth bass (*Micropterus salmoides*) revealed that maternal cortisol treatment prior to spawn also affected offspring phenotype; offspring of cortisol-treated females had higher masses right after hatch, had greater fear responses, were less bold and less anxious, and exhibited an attenuated cortisol response to an acute stressor. Together, the results of the present thesis suggest that effects of maternal stress prior to spawn on offspring survival, growth, responses to stress, and anxiety-related behaviours are mediated, at least in part, by elevated maternal cortisol but not likely via increased deposition of maternal cortisol into eggs. The effects of maternal stress and cortisol treatment on offspring reported in the present thesis also suggest that maternal stress may prime offspring with adaptive traits to better survive in a stressful environment.

RÉSUMÉ

Le but de cette thèse était de déterminer si le stress social maternel ou le traitement avec le cortisol, une hormone glucocorticoïde (GC) avant le frayage, affecte la réponse de stress et les comportements d'anxiété chez les descendants. Chez les poissons zèbres (*Danio rerio*), la progéniture de femelles ayant un statut social dominant se montrent plus audacieux à 6 jours après la fertilisation (JAF). Cependant, il est intéressant de noter que, lorsque comparé à la progéniture de femelles contrôles, les femelles qui ont pris part dans des interactions sociales (quel que soit le statut social atteint dans la hiérarchie de dominance) ont produit une progéniture qui avait une meilleure survie à 1 JAF, qui présentait des concentrations réduites de cortisol de base à 0 et 30 JAF, et qui démontrait une réaction de peur plus intense à 6 JAF, caractérisé par une plus grande diminution en natation active en réponse à un éclairage lumineux abrupte. De plus, une expérience de terrain comprenant l'achigan à grande bouche (*Micropterus salmoides*) a dévoilé que le traitement maternel de cortisol avant le frayage affectait le phénotype de la progéniture; les individus nés de mères ayant été traitées avec le cortisol étaient moins audacieux et aussi moins anxieux, ils avaient des masses plus élevées peu après l'éclosion et ils démontraient une réponse de cortisol atténuée suite à leur exposition à un stresseur aigu. Ensemble, les résultats de cette thèse suggèrent que les effets qu'a le stress maternel avant le frayage sur la survie, la croissance, la réponse au stress et les comportements anxieux de la progéniture sont en partie arbitrés par une élévation du cortisol maternel. Cependant, il est peu probable que ces effets ont été causés par un dépôt accru de cortisol maternel dans les œufs. De plus, les effets du stress maternel et du traitement au cortisol sur la progéniture présentés dans cette thèse suggèrent que le stress maternel pourrait armer la progéniture avec des traits adaptifs servant à améliorer leur survie dans un environnement stressant.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
11βHSD-2	11β-hydroxysteroid dehydrogenase type 2
ACTH	Adrenocorticotropic hormone
AIC	Akaike information criterion
ANOVA	Analysis of variance
CRF	Corticotropin-releasing factor
DNA	Deoxyribonucleic acid
DPF	Days post-fertilization
DPI	Days post-injection (of cortisol)
EIA	Enzyme-linked immunosorbent assay
ESF	Egg-sac fry
fps	Frames per second
FSF	Free-swimming fry
GC	Glucocorticoid
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
HPF	Hours post-fertilization
HPG	Hypothalamic-pituitary-gonadal
HPI	Hypothalamic-pituitary-interrenal
HPT	Hypothalamic-pituitary-thyroid
miRNA	Micro-ribonucleic acid
MR	Mineralcorticoid receptor
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methanesulfonate
MT	Maternal (cortisol) treatment group
ND	No difference
NSERC	Natural Sciences and Engineering Research Council of Canada
p-glycoprotein	Permeability glycoprotein
SEM	Standard error
YOY	Young-of-the-year

CHAPTER 1

GENERAL INTRODUCTION

All animals are exposed to stressors that can threaten lifetime fitness by reducing survival and reproductive success. Increasingly, research is demonstrating that stressors impact not only the individual, but the individual's offspring (Schreck et al., 2001; Green, 2008; Rodgers et al., 2013). Understanding how parental stress affects offspring fitness is relevant to conservation issues because the future of any population relies on the successful development, survival, and reproduction of its offspring. In particular, stress in female fish prior to spawn may affect offspring characteristics after hatch because maternal hormones, mRNA, proteins, and lipids are deposited into the yolks of developing eggs. The glucocorticoid (GC) cortisol, the main effector hormone of the teleost stress response, is of interest as a mediator of maternal stress on offspring characteristics because GCs play an organizational role during early development in teleost fish (Nesan and Vijayan, 2012, 2016). The present thesis examined the effects of maternal stress and cortisol-treatment as a proxy for maternal stress on offspring behaviour and physiology in two teleost fish, domesticated zebrafish (*Danio rerio*) and wild largemouth bass (*Micropterus salmoides*). The present chapter introduces the teleost stress response, provides a brief review of the documented effects of maternal stress and exogenous cortisol exposure on offspring, discusses the use of exogenous GC administration in research, introduces the offspring endpoints of interest in the present thesis, and describes the research goals and predictions. Chapters 2 and 3 document the observed impacts of maternal social status and maternal cortisol exposure on offspring characteristics in zebrafish and largemouth bass, respectively. The goal of both experiments was to test the hypothesis that maternal stress prior to spawn affects offspring phenotype, with a focus on offspring anxiety-related behaviour and the cortisol response to acute stress. The potential role that maternal cortisol can play in mediating such effects was examined.

1.1 Stress and the stress response in teleosts

The concept of stress has been widely used by biologists of all disciplines, from cell biologists to ecologists, and has been applied to explain observed phenomena at every level of organization (Wendelaar Bonga, 1997). In general, stress is described as a condition in which intrinsic or extrinsic stimuli (i.e. stressors) disturb the maintenance of homeostasis, a dynamic equilibrium within a system of living organisms (i.e. ecosystem) or within an individual (reviewed by Chrousos and Gold, 1992; Wendelaar Bonga, 1997). The present thesis focused on stress at the organismal level. In an effort to regain homeostatic balance following exposure to a stressor, animals initiate a stress response proportional to the magnitude of the stressor, where the stress response consists of a coordinated set of compensatory behavioural and physiological responses (Wendelaar Bonga, 1997).

The concept of stress historically was based largely on mammalian studies, but the vertebrate stress response is well-conserved and thus, the teleost stress response is similar to that of mammals (Wendelaar Bonga, 1997). Some physiological responses to a stressor can be specific to a particular type of stressor (Sánchez et al., 2011), but the stress response, regardless of the stressor, can be divided into three integrated phases (Wendelaar Bonga, 1997). The primary phase involves activation of the nervous and endocrine systems. The acute humoral adrenergic stress response (Perry and Capaldo, 2011), more popularly known as the "fight-or-flight" response (Canon, 1929), is characterized by the rapid release of catecholamines from chromaffin cells in the head kidney of teleost fish or the adrenal glands in non-teleost vertebrates. At the same time, the hypothalamic-pituitary-interrenal axis (HPI) in herpetofauna (Guillette et al., 1995) and fish (Wendelaar Bonga, 1997; Mommsen et al, 1999; Barton, 2002), or the HP-adrenal axis (HPA) in mammals (Reeder and Kramer, 2005) and birds (Siegel, 1980),

is activated. Activation of the HPI/A axis begins with the hypothalamic release of corticotropin releasing factor (CRF), which stimulates the corticotropes of the pituitary gland to secrete adrenocorticotropic hormone (ACTH), which then travels through the bloodstream to the head kidney/adrenal glands, where it stimulates the synthesis of GCs by steroidogenic cells (Fig. 1.1). The primary GC hormone is cortisol in fishes as well as most mammals other than rodents, while corticosterone is the primary GC in rodents, birds, and herpetofauna (Bury and Sturm, 2007). Behaviour (Schreck et al., 1997; Gregory & Wood, 1999), as well as physiological processes including reproduction (Pankurst and Van Der Kraak, 1997; Schreck et al., 2001), metabolism (Fletcher, 1997; Vijayan et al., 1997; Mommsen et al., 1999), and immune function (Pickering and Pottinger, 1985; Balm, 1997) all can be affected by the onset of the stress response and/or GCs. Whereas catecholamine levels rise and peak within the first few minutes (min) following stressor exposure, GC levels rise and fall more slowly (Wendelaar Bonga, 1997). Given the slower time course of GCs in comparison to catecholamines, and the variety of tissues from which GCs can be extracted in vertebrates (e.g. plasma, saliva, hair/fur/skin/feathers, feces, urine; Sheriff et al., 2011), the most commonly measured trait assessing the extent to which an animal mounts a stress response is the concentration of GCs (Cooke and O'Connor, 2010).

The rise in cortisol induces the onset of the second phase of the stress response, during which the adrenergic and GC stress hormones activate physiological processes that help the animal address the challenge or threat (Wendelaar Bonga, 1997). Cortisol acting via GC or mineralcorticoid receptors (GR and MR, respectively) elicits widespread physiological responses that mobilize energy stores to allow the fish to cope or escape the stressor and regain homeostasis (Wendelaar Bonga, 1997). For example, exposure to an acute stressor typically suppresses maintenance functions (e.g. decreases bone formation; Chyun et al., 1984), but

increases metabolic (Barton and Schreck, 1987) and respiratory rates (Vaughan et al., 1982), permeability of the gills to water and ions (Wendelaar Bonga, 1997), and gluconeogenesis (Vijayan et al., 1991). Effects of the secondary stress response on immune function depend on the cell type; exposure of the flounder *Limanda limanda* to an acute stressor resulted in increases in circulating leukocytes (e.g. phagocytes) but decreases in circulating lymphocyte numbers (Pulsford et al., 1994).

The fact that the action of the acute stress response (i.e. HPI/A axis activation) is so well conserved among vertebrates highlights the importance of optimal GC management (Boonstra, 2005). Indeed, it is an evolutionarily important trait because of its potential to be an adaptive trait: the responsiveness of an individual's stress axis is repeatable under consistent conditions (Cook et al., 2011), there is significant variation among individuals within a species (Williams, 2008), populations show a change in the ratio of high vs. low responders in response to selective pressures (e.g. captivity; Evans et al., 2006), and a greater cortisol response to stressors has been negatively correlated with survival (MacDougall-Shackleton et al., 2009) and therefore is relevant to fitness. Lastly, the acute stress response is also, at least in part, a heritable trait (Pottinger and Carrick, 1999; see section 1.6).

If an individual is exposed to an acute or short-term stressor (e.g. handling stress; Pickering et al., 1982), the actions of GC-mediated secondary responses generally allow the stressed animal to cope with or escape the acute stressor. However, if an individual is exposed to a chronic stressor, which either is a prolonged single stressor (e.g. heat; Pérez-Casanova et al., 2008) or repeated exposure to an acute stressor (e.g. being chased twice daily for several days; Sopinka et al., 2014), then the third phase of the stress response will be initiated.

The third phase functions at the whole-organism level and is characterized by altered behaviour along with decreased growth rate, reproduction, and immune function (Pickering et al., 1982; Pickering and Pottinger, 1989). The first two phases of the stress response are considered to be adaptive to allow the stressed individual to cope with/escape an acute stressor, but an individual's response to chronic stress (i.e. the third phase of the stress response) may become maladaptive (reviewed by Wendelaar Bonga, 1997). Chronic stress in vertebrates can lead to a prolonged inhibition of growth (DiBattista et al., 2006), reproduction (Moore et al., 1991), and immune function (reduced antibody production and slower wound healing; Barcellos et al., 2004; reviewed by Wendelaar Bonga, 1997), as well as cell death of neurons (Bachis et al., 2008) and branchial epithelial cells (Wendelaar Bonga and Lock, 1992), reduced metabolic scope (Lankford et al., 2005), and increased oxidative stress (Lucca et al., 2009) and fat deposition (Rebuffé-Scrive et al., 1992). Behaviourally, chronic stress has been associated with reduced aggression, activity, and feeding (Gilmour et al., 2005), as well as disrupted parental care (Champagne and Meaney, 2006). The widespread nature of the effects of exposure to chronic stress suggests the involvement of many causal mechanisms. As the main effector hormone of the stress response in fish, cortisol has numerous targets both in the central nervous system and in peripheral tissues, and thus either is involved in or increases the responses to chronic/repeated stress (Sapolsky et al., 2000).

The dynamic process by which the body responds to stressors to regain homeostasis is termed allostasis, and the cumulative consequences of repeatedly or constantly responding to chronic stress can be measured as allostatic load (McEwan and Stellar, 1993). Such effects of chronic stress have been well-studied across taxa, but primarily within a single generation. The present thesis explored the intergenerational effects of chronic maternal stress.

Figure 1.1 A conceptual diagram (adapted from Sopinka et al., 2015) outlining the elevation of maternal cortisol either by endogenous production following a chronic stressor (solid lines) or by exogenous administration (dashed lines), and its potential effects on offspring phenotypes. First, a perceived stressor is transduced into a stress response via activation of the hypothalamic-pituitary-interrenal (HPI) axis, which begins with the production of corticotropin releasing factor (CRF) in the hypothalamus. This tropic hormone then stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary, which in turn stimulates cortisol production by the interrenal cells of the head kidney. Both cortisol and ACTH can inhibit further HPI axis action via negative feedback (dotted lines). Administration of exogenous cortisol bypasses activation of the HPI axis and results in sustained elevation of circulating cortisol concentrations, a result similar to the outcome of exposure to a chronic stressor. Regardless of the source of cortisol, sustained elevation of this GC hormone in female teleosts prior to spawn has been reported to affect many offspring traits, of which offspring survival, growth, anxiety-related behaviour, and cortisol responses to stress were assessed in the present thesis.

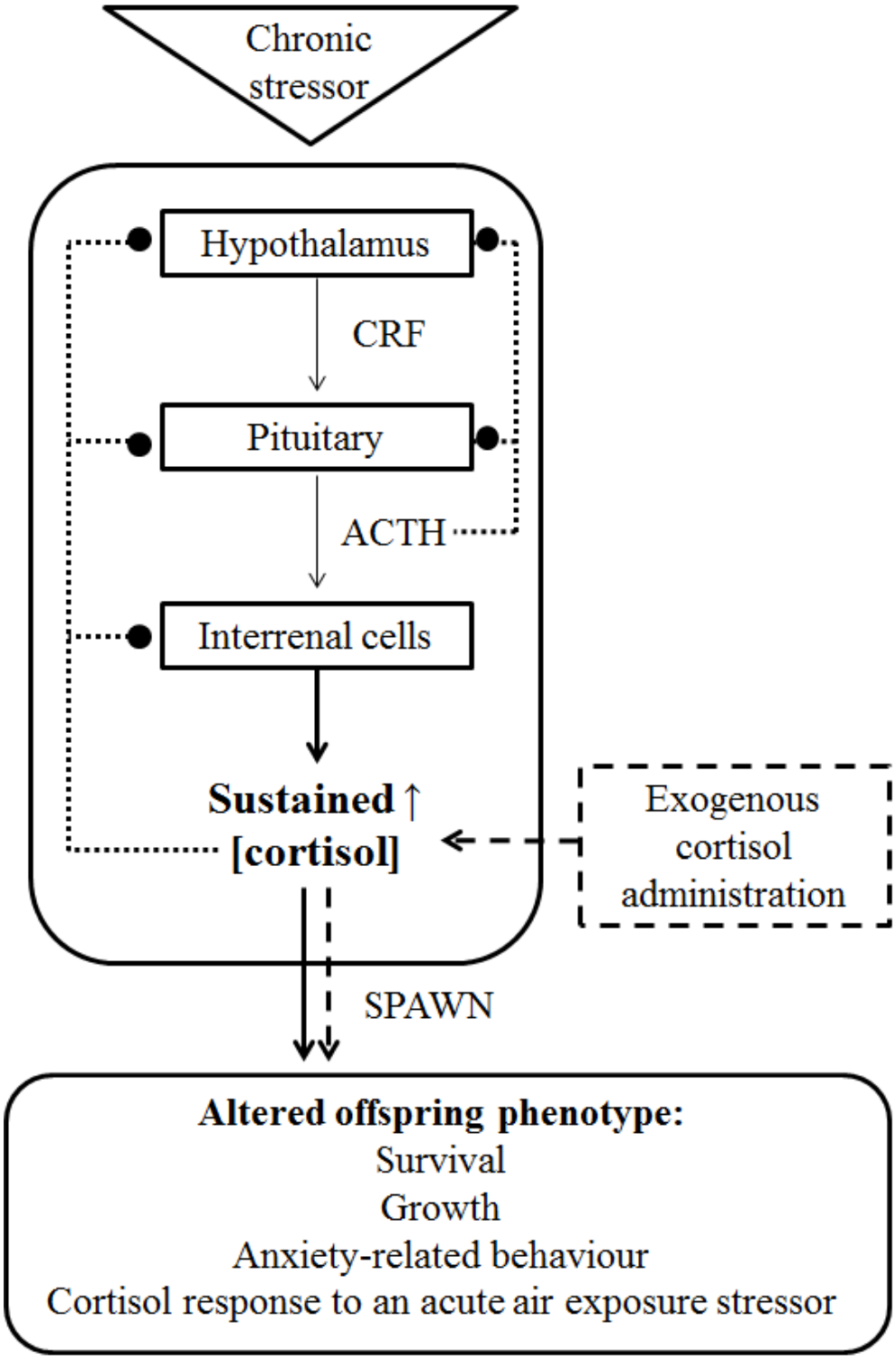
Chronic stressor
Hypothalamus
CRF
Pituitary
ACTH
Interrenal cells
Sustained ↑ [cortisol]
Exogenous cortisol administration
SPAWN
Altered offspring phenotype:
Survival
Growth
Anxiety-related behaviour
Cortisol response to an acute air exposure stressor

Figure 1.1

1.2 Offspring effects of maternal stress

Parental stress has the potential to alter offspring phenotype via "nature" and/or "nurture" according to when the parent is exposed to the stressor. Parental stress has been associated with reduced investment in offspring (i.e. reduced parental care or reduced maternal deposition of nutrients into the egg) as a trade-off in favour of (parental) survival and/or future reproductive success, with sustained effects on offspring fitness (Sheriff and Love, 2013). For example, in three-spined stickleback (*Gasterosteus aculeatus*), the male provides sole parental care to the brood, but stress in both parents can have long-term effects on offspring behaviour via epigenetic effects of reduced parental care (McGhee and Bell, 2014) and altered egg provisions (Mommer and Bell, 2013) from the male and female parent, respectively. In this case, maternal stress affected offspring nature, while paternal stress affected offspring nurture. The present thesis focused on how stress prior to fertilization can affect offspring phenotype (i.e. nature).

Intergenerational effects of stress prior to fertilization largely have been attributed to the female rather than the male parent because, although the male parent provides the offspring with half of the embryonic DNA, the female also provisions the developing embryo with yolk (placenta in mammals) containing maternal mRNA, proteins, lipids, and hormones (Mousseau and Fox, 1998). However, effects of paternal stress prior to fertilization on offspring no longer can be disregarded because a recent study by Rodgers et al. (2015) in mice discovered that paternal stress prior to breeding led to less anxious offspring, an epigenetic effect mediated by increased micro-RNAs (miRNA) packaged in sperm that silence maternal mRNA. Nonetheless, maternal stress prior to fertilization has been reported to have widespread effects on offspring (Sheriff and Love, 2013).

Most studies assessing the effects on offspring of maternal stress have been conducted in mammals, birds, and to a lesser extent, reptiles (reviewed by Sopinka et al., 2015). The first study in fish was published by Cloud in 1981, but 25 of the 36 published studies found in a recent search of the literature assessing effects of maternal stress (and proxies thereof, see section 1.3) on offspring in fish were conducted within the past ten years. Pre-spawn maternal stress in teleosts has been reported to affect reproductive success (e.g. timing of spawning and/or hatching, clutch size, etc.) as well as offspring survival, morphology, physiology, and behaviour (Table 1.1). Broadly, the reported effects of maternal stress include delayed spawning, smaller/lighter embryos, and reduced larval size, survival, responsiveness to stress, boldness, and ability to learn. However, such effects are variable in occurrence, raising the question, what are the sources of variation?

The effects of maternal stress prior to spawn on offspring phenotype seem to be context-specific, because studies assessing the same question in the same species may report contradictory results. For example, Giesing et al. (2011) and McGhee et al. (2012) reported increased and decreased anti-predator behaviour in offspring of female three-spined stickleback that were exposed to a predator stressor during oogenesis. Why the difference? Sopinka et al. (2015) argued that several possible sources of variation need to be taken into consideration when comparing results from different studies; effects of maternal stress depend on the type and intensity of stressor (or method of GC manipulation, see section 1.4), the duration of exposure, the environment in which the stressor occurs, the phase of oogenesis during which the female was exposed to the stressor, and the offspring life-stage assessed (elaborated in Appendix A). In the case of the apparently contradictory stickleback findings, the difference in anti-predator behaviour between the two studies could reflect a difference in offspring age tested (~100 day

difference), or the fact that Giesing et al. (2011) tested group anti-predator behaviour while McGhee et al. (2012) tested individual anti-predator behaviour.

Table 1.1 Summary of results from published literature assessing the effects of maternal stress prior to spawn on maternal reproductive success and offspring characteristics including survival, morphology, physiology, and behaviour in teleost fish. The direction of effect on a particular offspring trait is noted as ↑, ↓, or ND to indicate an increase, decrease, or no difference, respectively.

Table 1.1

TELEOST SPECIES	MATERNAL STRESSOR	CLUTCH SIZE & HATCHING	SURVIVAL	PHYSIOLOGY	MORPHOLOGY	BEHAVIOUR	REFERENCE
African cichlid (*Neolamprologus pulcher*)	Chasing and confinement	↑spawning delay ↓ clutch size			↓ embryo size and mass		Mileva et al., 2011
Brown trout (*Salmo trutta*)	Subordinate social status				↓ variation in size of siblings		Burton et al., 2013
Chum salmon (*Oncorhynchus keta*)	Interspecific competition		↓				Essington et al., 2000
Coho salmon (*Oncorhynchus kisutch*)	Chasing	ND hatching time	ND	↑embryo [cortisol]	ND growth rate ND body condition factor		Stratholt et al., 1997
Rainbow trout (*Oncorhynchus mykiss*)	Air exposure	↑spawning delay ND fecundity	↓		↓embryo size ND larval size		Campbell et al., 1992
	Confinement	ND fecundity	↓		↓embryo size ND larval size		Campbell et al., 1994
	Chasing, crowding, tank draining & noise	↓time to spawn ND fecundity	ND	ND in immune resistance to infection	↓embryo size ↓larval length		Contreras-Sanchez et al., 1998
Sockeye salmon (*Oncorhynchus nerka*)	Exercise deprivation		↓				Patterson et al., 2004
	Captivity	↓fertilization success					
	Chasing		ND	ND embryo [cortisol]	ND embryo size	↑bouts of burst swimming	Sopinka et al., 2014
				↓ cortisol response to acute stress ↓ rise in mRNA abundance of cortisol synthesis genes in head kidney after acute stress exposure			Sopinka et al., 2016

TELEOST SPECIES	MATERNAL STRESSOR	CLUTCH SIZE & HATCHING	SURVIVAL	PHYSIOLOGY	MORPHOLOGY	BEHAVIOUR	REFERENCE
Sockeye salmon (*Oncorhynchus nerka*)	Length of migration			ND oxidative stress ND antioxidant capacity			Braun et al., 2013
		ND fecundity			↓embryo size ↓larval mass		Taylor et al., 2015
	Intraspecific competition		↓				Essington et al., 2000
Striped trumpeter fish (*Latris lineata*)	Frequent handling	↓% of females spawned ↑fecundity					Morehead et al., 2000
Three-spined stickleback (*Gasterosteus aculeatus*)	Predator exposure			↑embryo [cortisol] ↑ embryo oxygen consumption	↑embryo size ND post-hatch growth	↑ anti-predator behaviour	Giesing et al., 2011
			↓			↓ anti-predator behaviour	McGhee et al., 2012
				ND cortisol responsiveness to stress			Mommer and Bell, 2013
				Altered expression of genes linked to metabolism, epigenetic inheritance, and neural proliferation and differentiation	↑embryo size ↑embryonic developmental rate		Mommer and Bell, 2014
						↓ ability to learn	Roche et al., 2012
Tropical damselfish (*Pomacentrus amboinensis*)	Intraspecific competition	ND clutch size		↑ embryo [cortisol]	ND yolk size ↓ larval size		McCormick, 2006
	Intraspecific competition & [egg] predator exposure			↑ embryo [cortisol]	↓ embryo size ↓ larval size		McCormick, 1998
		ND clutch size			↓ larval size		McCormick, 2009
Zebrafish (*Danio rerio*)	Subordinate social status	ND fecundity	ND	ND embryo [cortisol] ↑ expression of genes involved in cortisol synthesis ↓ stress-induced cortisol at 6 DPF			Jeffrey and Gilmour, 2016

1.3 Possible role of GCs in mediating effects of maternal stress on offspring phenotype

Female fish transfer maternal mRNAs, proteins, lipids, and hormones (including GCs) into the yolk sac of developing oocytes during the short vitellogenesis stage of egg development (Brooks et al., 1997; Faught et al., 2016). As a consequence of their lipophilic nature, steroid hormones like GCs are thought to move passively from maternal circulation into eggs (Groothuis and Schwab, 2008), but the mechanism of transfer is still under debate, with Moore and Johnston (2008) proposing a regulated process of deposition. Regardless, the physiological status of the female is crucial during this window of yolk "stocking" because stressed females may alter the amount or ratio of the different egg yolk components, with possible effects on development and/or developmental rate that may have long-term effects after hatch (Brooks et al., 1997). Maternal stress and its associated elevation of circulating cortisol levels may affect offspring phenotype through altered yolk provisions (protein, lipid, GCs and other hormones), increased action of barriers to diffusion (e.g. conversion of cortisol to its inactive metabolite cortisone), or epigenetic effects on offspring gene expression.

First, given the inhibitory effect of stress on reproductive investment (e.g. smaller clutch size and embryo volume/mass, Mileva et al., 2011; reviewed by Wendelaar Bonga, 1997), it is perhaps not surprising that maternal stress may affect offspring by altering maternal investment of nutrients into eggs/embryos. Most previous studies that tested the effects of elevated maternal cortisol on overall embryo diameter/volume and mass (i.e. including yolk) reported a reduction in nutrient deposition (i.e. decreased embryo size/mass; Contreras-Sanchez et al., 1998; Mileva et al., 2011; Table 1.1), while a minority reported no difference (McCormick, 2006; Sopinka et al., 2014) or an increase (Giesing et al., 2011; Mommer and Bell, 2014) in size/mass. Whether a reduced embryonic size/mass is reflective of a smaller yolk or organism (or both) cannot be

determined unless the yolk is detached and measured separately (McCormick, 2006). Regardless of the portion of the embryo that is smaller, reduced embryo size/mass is often translated into reduced larval size/mass (McCormick, 1998; Contreras-Sanchez, 1998; Taylor et al., 2015), most likely owing to the smaller bank of yolk resources from which the developing offspring draws nutrients for growth.

Second, other offspring provisions to the egg that can be affected by maternal stress include lipid-soluble hormones. The yolk sac of a fish embryo typically contains several lipid-soluble hormones including cortisol, the thyroid hormones thyroxine and triiodothyronine, and the sex steroids estradiol and testosterone (de Jesus and Hirano, 1992). In addition to playing specific roles in growth, osmoregulation, development, and reproduction, both the thyroid and sex hormones may impact the stress response in fish, reflecting the integration of the HPI axis with the HP-thyroid (HPT) and the HP-gonadal (HPG) axes (reviewed by Wendelaar Bonga, 1997; Bernier et al., 2009). Consequently, the disturbance of one axis often leads to a change in the others. For example, HPI activation in response to a stressor tends to suppress the HPG axis while increasing action of the HPT axis (reviewed by Wendelaar Bonga, 1997; Peter, 2011). As such, chronic maternal stress during vitellogenesis has the potential to alter the yolk deposits not only of cortisol, but also of sex and thyroid hormones. These, in turn, may influence offspring development. For example, hens (*Gallus gallus domesticus*) with experimentally elevated plasma GCs laid eggs with lower concentrations of progesterone and testosterone, as well as smaller egg and yolk mass (Henriksen et al., 2011). Conversely, experimentally increased yolk testosterone was associated with increased aggression, growth, immunity, and survival in chicken hatchlings (reviewed by Riedstra et al., 2012). An increase in yolk thyroid hormones was linked to increased body length and survival (Ayson and Lam, 1992).

The deposition of cortisol into the egg is positively correlated with the concentration of circulating maternal cortisol in at least some teleost species (Stratholt et al. 1997; Eriksen et al. 2006; 2011; Mingst et al., 2007; but see Faught et al., 2016). Maternal cortisol deposition is therefore the third possible mechanism potentially involved in mediating effects of elevated maternal cortisol on offspring phenotype before and after hatch. Embryonic cortisol plays an important role during vertebrate development; it interacts with other developmental hormones such as growth hormone, thyroid and sex hormones (Mathiyalagan et al., 1996; Brown and Kim, 1995; Schreck et al., 2001). Most importantly, cortisol has been reported to play an organisational role in many early developmental pathways in fish, including development of the eye and HPI axis, the formation of skeletal and cardiac muscle, and neurogenesis (Nesan et al., 2012; Nesan and Vijayan, 2012; 2016; Pikulkaew et al., 2011).

Although some studies in which females were exposed to chronic stress prior to spawn can attribute observed effects on offspring (at least in part) to higher deposition of cortisol into eggs (McCormick, 1998; 2006), other studies failed to observe differences in egg/embryo cortisol levels (e.g. Mileva et al., 2011; Sopinka et al., 2014; Jeffrey and Gilmour, 2016). However, even when elevated maternal cortisol did not translate into elevated egg cortisol concentrations, maternal stress was reported to affect offspring behaviour (Sopinka et al., 2014) and physiology (Jeffrey and Gilmour, 2016), suggesting that offspring programming by maternal stress is not solely mediated by cortisol. Even where significantly higher cortisol content was found in eggs of stressed females compared to eggs of unstressed females, egg cortisol concentrations were not proportional to ovarian fluid cortisol levels. For example, Contreras-Sanchez et al. (1998) noted that rainbow trout ovarian fluid and egg cortisol concentrations were 16.6 and 30.5-fold lower, respectively, than plasma cortisol concentration. This difference

suggests the presence of a maternal mechanism to avoid egg hypercortisolism. One mechanism that has been proposed (Faught et al., 2016) is an increase in activity of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD-2), which catalyses the conversion of cortisol to its inactive form cortisone (Mommsen et al., 1999). In zebrafish, cortisol deposition into the developing oocyte yolk is regulated at least in part by ovarian 11βHSD-2 (Faught et al., 2016). Specifically, ovarian follicle 11βHSD-2 levels increased 7-fold in tissue treated *in vitro* with cortisol, suggesting tight regulation of cortisol deposition by ovarian follicles (Faught et al., 2016). A second possible barrier to cortisol diffusion could be increased permeability glycoproteins (p-glycoproteins), which are membrane transporters known to prevent the equilibration of hydrophobic substances (such as steroid hormones) across membranes (Painter and Moore, 2005). Unlike 11βHSD-2, p-glycoproteins have not been studied in the context of maternal stress in fish; however, they warrant investigation because p-glycoproteins are known to be present in the ovary of fish (Bard, 2000).

A fourth possible mechanism through which maternal stress may affect offspring involves the deposition of maternal mRNA during vitellogenesis (Mommsen et al., 1999). Differences in maternal transcript abundance in the egg, in turn, could have direct or indirect effects on offspring. For example, increased transfer of maternal mRNA for the GR could directly amplify the effects of maternal cortisol also deposited into the egg. Alternatively, maternal stress prior to spawn may lead to indirect or epigenetic effects on offspring, i.e. changes in gene expression without alterations of DNA sequence. For example, maternal stress in rats was associated with increased methylation of a promoter region of GR, which decreased the expression of GR in the offspring (Szyf et al., 2005). Less GR could limit the amount of cortisol that can bind, thus leading to reduced responsiveness to stress, or alternatively, the lowered

binding of cortisol to GR could reduce negative feedback and result in the continued production of GC and thus higher responsiveness to stress (Weaver et al., 2004). In fish, maternal stress in three-spined stickleback led to increased expression of DNA methyltransferase, the enzyme responsible for DNA methylation (Mommer and Bell, 2014), and increased egg cortisol in zebrafish was associated with decreased expression of the HPI axis genes and an attenuated cortisol response to stress (Nesan and Vijayan, 2016). Taken together, these observations suggest that maternal stress in fish may function to attenuate the stress response of offspring via an epigenetic mechanism.

In summary, the mechanisms through which maternal stress prior to spawn may alter offspring phenotype are numerous and not mutually exclusive. The proposed maternal GC-modulated mechanisms may result in permanent organisational effects on offspring development (e.g. direct GC deposition into eggs causing deformities in heart development; Nesan and Vijayan, 2012) or activational effects (i.e. GCs affecting a system that has already developed; Love et al., 2013; e.g. smaller yolk sac leading to reduced larval growth; Eriksen et al., 2006). Williams (2008) argues that organisational effects have greater, longer-term effects on fitness. However, regardless of the specific mechanism of action, many offspring effects of maternal stress appear to be affected by the elevation of maternal cortisol following activation of the HPI axis during egg development.

1.4 Exogenous GC administration as a proxy for maternal stress

Experimental manipulation of GCs is a common technique in stress research because it reduces sources of inter- and intra-individual variation and for logistical reasons in the context of

field work under time constraints. A working model of the stress response involves three steps: perception, transduction and response (Wingfield and Mukai, 2009). First, an organism must perceive a change in the environment through some sensory modality (e.g. vision), and then transduce that information via the central nervous system into activation of the neuroendocrine or primary phase of the stress response, thus resulting in increases in circulating catecholamine and/or GCs; these hormones ultimately alter the stressed individual's molecular/physiological/behavioural phenotype to be able to cope with the stressor (Wingfield and Mukai, 2009; Wendelaar Bonga, 1997). Importantly, variation in responsiveness to a stressor exists at each of these steps because individuals vary in the intensity of their responses to a particular stressor (Sapolsky, 2000; e.g. depending on time of day; i.e. intra-individual variation), and variation between individuals in a population also naturally exists (i.e. inter-individual/intra-specific variation; Williams, 2008). As such, it can be difficult for researchers to draw conclusions when studying the causal effects of stressor exposure on organismal responses. Consequently, many biologists have opted to by-pass the perception and transduction stages of the stress response in favour of manipulating the physiological response via experimental administration of exogenous GCs (Fig. 1.1). This alternative approach mimics the end result of HPI/A axis activation (i.e. sustained elevation in circulating GCs) in a standardized manner (via dosage) while minimizing variation associated with stressor perception and transduction (reviewed by Sopinka et al., 2015). Studies testing the effects of GC manipulation in parallel with exposure to a stressor provide valuable insight into whether (and to what extent) GCs are involved in eliciting a specific response to a stressor. That is, if both exogenous GC administration and stressor exposure produce similar phenotypes in test subjects, then elevated

GCs as a result of HPI/A axis activation can be deemed a causal factor in producing the phenotype (e.g. McCormick, 1998).

It should be noted that standardizing GC dosage by subject weight can account for some but not all variation in the downstream effects of elevated GCs, such as individual differences in GR density and negative feedback within the HPI/A axis (Sapolsky, 2000; Fusani, 2008; Romero, 2004). Sopinka et al. (2015) reviewed the methods and uses of GC manipulation and warned of several factors that should be considered when designing experiments involving this experimental technique. First, to ensure ecological and physiological relevance, studies that experimentally manipulated GCs should validate the chosen dose via dose response curves. In other words, the rise in circulating GCs post-treatment should lie within a physiologically relevant range. For example, an injection of cortisol, suspended in a coconut butter vehicle, into the peritoneal cavity of wild creek chub (*Semotilus atromaculatus*) caused a prolonged (3 day) rise in plasma GC concentrations to a level similar to the endogenous levels achieved following exposure to a standardized chasing stressor (Nagrodski et al., 2013). Also, the method of GC administration should be chosen based on the type of stressor that is of interest. Two factors determine the duration of GC-elevation: treatment frequency and GC delivery medium. Depending on the delivery medium, a single treatment elicits a transient elevation of circulating GCs comparable to an acute stress response, whereas repeated treatment leads to sustained elevation in circulating GCs comparable to a chronic stressor (Sopinka et al., 2015). Similarly, a fast-release coconut oil injection compared to a slow release cocoa butter implant elicits, respectively, a transient vs. sustained elevation in circulating GCs in fish. With appropriate validation, GC manipulation is an invaluable experimental technique because it avoids variation associated with stressor perception and transduction, it standardizes the elevation in GC levels,

and it provides the opportunity to determine the proximate mechanism underlying an organism's response to a stressor.

Just as experimental GC manipulation can provide insight into the mechanisms through which stressed animals cope, it also can be used to assess how elevated GCs impact future generations. One approach is to directly treat the eggs of oviparous species with exogenous GCs via hormone injection or bathing. However, Sopinka et al. (2016) point out that this logistically simple method of elevating offspring GCs does not account for possible effects of maternal buffering (Li et al., 2012) or epigenetics (Ho and Burggren, 2010). As discussed above, deposition of maternal cortisol into the egg yolk is one of several candidate mechanisms that may be responsible for effects of maternal stress on offspring; other possible, non-mutually exclusive mechanisms include actions of 11βHSD-2, epigenetics, or altered egg provisions. As such, experimental elevation of maternal GCs rather than direct exposure of eggs may be a more appropriate proxy for maternal stress. In teleosts, both direct (treatment of eggs) and indirect (treatment of females prior to spawn) GC manipulation can affect offspring traits (Table 1.2). Overall, exogenous cortisol administration to females prior to spawn or directly to eggs tends to result in morphological malformations (Nesan and Vijayan, 2016; Eriksen et al., 2006), an attenuated cortisol response to acute stress (Auperin and Geslin, 2008; Nesan and Vijayan, 2016), and offspring that are less aggressive and active behaviourally, and generally exhibit a more subordinate or reactive stress coping style (see section 1.7; Wilson et al., 2013; Eriksen et al., 2011; Burton et al., 2011). Other endpoints that have been assessed offer mixed results (Table 1.2). For example, elevated maternal/egg cortisol has been reported to either increase or decrease egg yolk size (e.g. ↑ in McCormick, 1999 but ↓ in McCormick, 1998) and larval offspring length (e.g. ↑ in Nesan and Vijayan, 2016 but ↓ in McCormick, 1998), and to have

either no effect on offspring survival or to correlate negatively with offspring survival (e.g. no effect in Sloman, 2010 but ↓in McConnachie et al., 2012). Similar results were obtained for metabolic rate (e.g. no effect in Burton et al., 2011 but ↑ in Eriksen et al., 2006). Regardless, it is important to consider whether observed effects are adaptive for offspring so that an ecological and evolutionary perspective on population dynamics may be gained (Gagliano and McCormick, 2007).

Most studies that have assessed the effects of maternal stress on offspring report that maternal stress is harmful to offspring. This statement is particularly true for biomedical research using traditional rodent and human models (Weinstock, 2005; 2008). Some studies conducted with fish have reported similar trends, where offspring of stressed or cortisol-treated females suffered poorer survival (Stress: McGhee et al., 2012; Patterson et al., 2004; Essington et al., 2000; Campbell et al., 1992, 1994; GC manipulation: Eriksen et al., 2006). Conversely, a growing body of literature suggests that stressed adults may prime their offspring with adaptive phenotypic traits to better handle future stressors reflective of the parents' environment (Mousseau and Fox, 1998). This hypothesis of transgenerational adaptive plasticity posits greater offspring fitness and therefore greater parental reproductive fitness (Green, 2008). For example, offspring of predator-exposed three-spined stickleback females displayed tighter shoaling behaviour (Giesing et al., 2011). Increased anti-predator behaviour should increase offspring survival and thus fitness, but only if the environment of the progeny also has a high risk of predation. By contrast, if the predation risk of the offspring's environment does not match the high risk of the maternal environment, then the heightened vigilance exhibited by the offspring may be unnecessary and potentially maladaptive owing to its associated energetic cost. According to this maternal match/mismatch hypothesis (Breuner, 2008; Love et al., 2013; Sheriff

and Love, 2013), even apparently negative traits may in fact prove to be adaptive once the ecological context is taken into consideration. With this perspective in mind, the common observation of reduced larval growth in offspring of stressed mothers (see Tables 1.1 and 1.2) could be considered beneficial if food resource availability is limited, allowing those with lower growth rates to out-perform larger, faster-growing individuals because the latter cannot attain sufficient resources to support their higher growth rate (Love and Williams, 2008). Thus, the ecological context and the maternal match/mismatch hypothesis must be considered when determining whether the effects of maternal stress on offspring are adaptive.

Table 1.2 A summary of results from published literature on the effects of maternal (intraperitoneal injection and spiked food) or offspring (egg bath and embryonic microinjection) exogenous cortisol manipulation on maternal reproductive success and offspring characteristics including survival, morphology, physiology, and behaviour in teleost fish. The direction of effect on a particular offspring trait is noted as ↑, ↓, or ND to indicate an increase, decrease, or no difference, respectively.

Table 1.2

TELEOST SPECIES	CLUTCH SIZE & HATCHING	SURVIVAL	PHYSIOLOGY	MORPHOLOGY	BEHAVIOUR	REFERENCE
i) Maternal intraperitoneal injection						
Atlantic salmon (*Salmo salar*)		↓	↑egg [cortisol] ↓rate of yolk sac utilisation	↓yolk sac size ↓larval size & mass ↑deformities		Eriksen et al. 2006
					↓foraging success ND in propensity to achieve dominant status ↓aggression ↑time spent frozen	Eriksen et al. 2011
Pink salmon (*Oncorhynchus gorbuscha*)	↓fecundity					McConnachie et al., 2012
Tropical damselfish (*Pomacentrus amboinensis*)				↓yolk sac size ↓larval size		McCormick, 1998
ii) Spiked maternal food						
Zebrafish (*Danio rerio*)	↑fecundity		ND in embryo [cortisol] (i.e. no chronic elevation)			Faught et al., 2016
iii) Egg bath						
Asian seabass (*Lates calcarifer*)		↑in high salinity				Sampath-Kumar et al., 1993

TELEOST SPECIES	CLUTCH SIZE & HATCHING	SURVIVAL	PHYSIOLOGY	MORPHOLOGY	BEHAVIOUR	REFERENCE
iii) Egg bath continued						
Brown trout (*Salmo trutta*)			↑embryo [cortisol] ND in metabolic rate	↓larval length and mass ND in body condition	↓aggression ↓propensity to achieve dominant status	Burton et al., 2011
	ND hatch rate	ND	↑embryonic oxygen consumption rate ↑ammonia but ND in urea excretion rates	↑larval mass	↑aggression ↓ability to learn	Sloman, 2010
Medaka (*Oryzias latipes*)	↓ hatch rate			ND in growth		Cloud, 1981
Rainbow trout (*Oncorhynchus mykiss*)			Transient ↑ embryo [cortisol] ↓stress-induced [cortisol]			Auperin and Geslin, 2008
Sockeye and chum salmon (Oncorhynchus *nerka* & *O. keta*)			↑embryo [cortisol]	↑shape suitability to swimming environment	↓burst swimming velocity (not duration)	Sopinka, 2015
Tropical damselfish (*Pomacentrus amboinensis*)	↓ hatch rate	↓ emb but ↑ larval		↑larval asymmetry		Gagliano and McCormick, 2009
				↑egg yolk size ↓larval length		McCormick, 1999
Zebrafish (*Danio rerio*)	↑hatching success				ND distance travelled or average velocity ↑thigmotaxis	Wilson et al., 2013
			↓resting heartbeat but ↓stress-induced heartbeat	↓heart deformities		Nesan and Vijayan, 2012
iii) Embryonic microinjection						
Zebrafish (*Danio rerio*)			↓ rise in stress-induced [cortisol]; ↑baseline but ↓ after 60 min recovery ↓expression of HPI axis genes	↑cardiac edema ↑larval size at hatch		Nesan and Vijayan, 2016

1.5 Anxiety-related behaviour

Altered behaviour is a possible tertiary response to a stressor (Barton, 2002). The main function of both fear- and anxiety-related responses is to allow the individual to appropriately respond to a perceived threat (e.g. escape, avoidance, or defensive behaviour). Fear- and anxiety-related behaviours are often accompanied by (and arguably preceded by) increased autonomic and/or neuroendocrine (i.e. HPI/A axis) activation (reviewed by Steimer, 2002). Although the terms 'fear' and 'anxiety' are sometimes used interchangeably owing to similarities in behaviour, there are clear distinctions based on the type of threat. Anxiety is defined as a response to an unknown threat or internal conflict, whereas fear is a response to a known external danger (Craig et al., 1995). Specifically, the type of action taken to respond to a particular threat (e.g. predator proximity) depends on whether the individual is "uncertain" of the potential outcomes in a novel environment (i.e. fear of the unknown; Steimer, 2002), sensing a potential threat (e.g. being located in an area in which a previous encounter with a threat was experienced), sensing/visualizing an actual threat, or experiencing a threat/imminent attack (Fanselow, 1994). Perception of the former two elicits an anxiety response, while the latter two dangerous scenarios elicit a fear response. Put simply, any behaviour classically categorized as a fear response is considered an anxiety-related behaviour when it is exhibited in the absence of a true external imminent threat. Another notable difference between fear and anxiety is the duration of response. That is, an individual quickly ceases a fear-related behaviour once the danger has passed, whereas anxiety is a sustained state caused by the potentially prolonged anticipation of all possible future dangers (Davis et al., 2010).

In the present thesis, offspring anxiety was in general assessed rather than offspring fear for two reasons: to reduce experimental error; and because anxiety has a larger potential to affect

an individual's fitness. First, individuals perceive the level of danger of an external threat differently based in part on past experience, thus adding variation to tests assessing fear responses. Because tests of anxiety are void of such external stimuli, inter-individual differences in behaviour can be attributed to differences in innate anxiety (when all other factors are held constant). Second, the known negative effects of allostatic overload mean that prolonged anxiety responses accompanied by chronic HPI/A axis activation can be maladaptive. Also, fear and anxiety are not mutually exclusive, because anxiety can impair adaptive fear responses. For example, Jesuthasan (2012) observed that when adult zebrafish with low innate anxiety levels encountered a stimulus perceived as an immediate threat (i.e. presence of a predator), they would exhibit an appropriate and adaptive fear response by darting away from the stimulus. However, when zebrafish with high innate anxiety levels encountered the same threat, they tended to freeze, thus decreasing their probability of survival. Indeed, high anxiety levels, and the resulting impairment of fear responses, have been positively correlated with neural aging (e.g. increased rate of neurodegeneration with impaired neurogenesis; Perna et al., 2016). The demonstration of adaptive fear-related escape behaviour was similarly negatively correlated with maladaptive anxiety-related freezing behaviour in rodents (Mongeau et al., 2003). In summary, assessing anxiety-related behaviours is more reliable than assessing fear, and also more applicable to predicting lifetime survival and thus the fitness of an individual.

Tests of anxiety in fish generally involve recording the activity and differential distribution of exploratory behaviour in "threatening" versus "safe" areas of a testing chamber. The most common place preference tests for assessing anxiety-related avoidance behaviours in fish include the open-field test, the light-dark preference test, and more recently, the novel tank diving test, all of which were adapted for fish within the last 20 years from the commonly used

open-field test conducted with rodents in preclinical studies as many as 50 years ago (Denenberg, 1969). These three tests quantify three different anxiety-related behaviours; thigmotaxis, scototaxis, and geotaxis, defined as the tendency for an individual to self-situate along the walls of the testing chamber, in dark environments, and at the bottom of the water column, respectively (reviewed by Schnörr et al., 2012; Maximino et al., 2010; Egan et al., 2009). Behaviour can be validated as being indicative of anxiety if pharmacological studies report that anxiogenic drugs (e.g. caffeine) and anxiolytic drugs (e.g. diazepam) enhance and attenuate the behaviour, respectively (e.g. Steenbergen et al., 2011). Thigmotaxis, a Greek term meaning 'touch-loving' or more commonly referred to as "wall-hugging" (Schnörr et al., 2012), is an evolutionarily conserved behaviour and has been documented in both adult and juvenile fish (Maximino et al., 2010; Schnörr et al., 2012), rodents (Denenberg, 1969; Treit and Fundytus, 1988), and humans (Kallai et al., 2007). Studies using the black-white preference test reported contradicting results. Whereas most published studies reported the display of scototaxis, a "dark-loving" behaviour in fish (Maximino et al., 2010, 2011), one study reported strong dark-avoidance behaviours in larval zebrafish (Steenbergen et al., 2011). This apparent discrepancy highlights the need to take life-history stage into account. It is hypothesized that adult fish prefer the dark half of the black-white preference chamber because the camouflage it offers provides protection from aerial predators (Thompson et al., 2016). If this is the case, then it is perhaps not surprising that larval zebrafish, which are small and transparent, may not display scototaxis because they would not benefit from camouflage. The third test of anxiety, the novel tank diving test, was first conducted by Levin et al. (2007) to assess geotaxis (Greek term meaning "bottom loving") in adult zebrafish, where geotaxis is an anxiety-related behaviour characterized by the tendency of a fish to seek protection in a novel environment by diving down until it becomes

bold enough to explore the remainder of the exposed water column (Levin et al., 2007; Sackerman et al., 2010; Maximino et al., 2010; Egan et al., 2009). To date, this test has not been used to assess anxiety in larval fish, so the present thesis describes the first characterization of larval fish behaviour during the novel tank diving test. This test is conceptually similar to the open-field test, but assesses vertical movement whereas the open-field test quantifies horizontal movement. All three tests also can be used to measure activity (distance travelled and/or average swimming velocity), as well as the personality trait of boldness. Boldness, which is negatively correlated with anxiety (Benning et al., 2005), is measured as the latency for a subject to leave the safe refuge area (i.e. deep, dark, outer area along the wall) and explore the more exposed and potentially dangerous novel areas.

Larval zebrafish exhibited anxiety-related behaviours as early as 4 DPF (Colwill and Creton, 2011). For adult and juvenile fish, automatic motion tracking software such as IdTracker (Pérez-Escudero et al., 2014) can reliably measure spatial, social, and kinematic behavioural characteristics. Unfortunately, the behaviour of larval fish is more complex to track because most tracking programs determine the location of a test subject by contrasting each video frame to a background reading (e.g. IdTracker; Pérez-Escudero et al., 2014; reviewed by Martineau and Mourrain, 2013) to determine the planar X-Y coordinates of the 'centre of mass' (i.e. centroid; Cario et al., 2011), and larval fish (especially zebrafish) have transparent bodies with two small dark eyes, so there are only a few dark pixels from which to "find" the fish. As such, larger shadows or ripples in the tank water can be mistaken for a larva when using common tracking programs, leading to erroneous location output. To date, the existing programs that have been optimized to track the motion of larval fish require larvae to be individually placed in small wells of 24- and 96-well microplates with a maximum water depth of 1 cm (Cario et al., 2011;

DanioVision, Noldus Information Technology), a format that is consistent only with the open-field test.

In summary, anxiety is characterized by an array of behaviours, and as such, can be reliably quantified by several tests and compared across different contexts to assess stress-coping style (see section 1.7). From the limited number of studies that have assessed the effects of maternal stress or elevated maternal/egg cortisol on offspring behaviour (Tables 1.1 and 1.2), maternal stress or elevated cortisol levels were positively correlated with offspring anxiety characterized by the increased display of thigmotaxis (Wilson et al., 2013) and time spent frozen (Eriksen et al., 2011).

1.6 GC response to acute stress

Pairing behavioural observations with physiological measurements is mutually beneficial to gain insight into the potential physiological mechanisms mediating observed behaviours, while at the same time observing whole-organism responses to altered physiological pathways and the resulting interaction with the environment. When studying anxiety-related behaviour in particular, the cortisol response to an acute stressor is a valuable physiological endpoint (Egan et al., 2009) because anxiety-related behaviours often are associated with increased autonomic and neuroendocrine activation (Steimer, 2002). In other words, the documented increase in anxiety-related behaviours in offspring of stressed females (Table 1.1) might be expected to be accompanied by greater HPI axis activation and thus a heightened cortisol response to acute stress in offspring of stressed females. Interestingly, this prediction does not seem to be met in

fish. Indeed, an attenuated cortisol response to an acute stressor has been reported in both offspring of stressed mothers and offspring hatched from cortisol-treated eggs (Jeffrey and Gilmour, 2016; Auperin and Geslin, 2008; Nesan and Vijayan, 2016). According to Love and Williams' (2008) maternal match hypothesis, if the environment experienced by the offspring matches the maternal environment in stress experienced, an attenuated stress response would be predicted to be adaptive by preventing constant and/or repeated stress axis activity and the associated physiological costs (e.g. GC-mediated gluconeogenesis).

Importantly to the present thesis concerning intergenerational effect of stress, the magnitude of an individual's cortisol response to an acute stressor is heritable. For example, in rainbow trout, 41% and 27% of the variation in responsiveness were explained by the responsiveness of the female and male parent, respectively (Pottinger and Carrick, 1999). The remaining 32% of the variation in offspring responsiveness to stress was not explained by parental responsiveness, and may reflect other factors such as egg GC levels; for example, trout hatched from cortisol-treated eggs displayed an attenuated stress response (e.g. Auperin and Geslin, 2008). In zebrafish, a decrease in the cortisol response to an acute stressor was documented in parallel with altered HPI axis gene expression in offspring of stressed females (Jeffrey and Gilmour, 2016) and offspring hatched from cortisol-treated eggs (Nesan and Vijayan , 2016).

1.7 Stress coping styles

An individual's stress coping style is characterized by its expression of a set of behavioural and physiological traits that are inter-correlated and consistently displayed over time

and across situational contexts in response to a true or expected stressor (Coppens et al., 2010). The term 'stress-coping style' often is used interchangeably with animal personality and behavioural syndromes (Sih et al., 2004), but the latter terms apply to a broader range of traits (e.g. shy-bold, exploratory behaviour, aggressiveness) and do not take into consideration the correlated neuroendocrine parameters that are consistently associated with certain behaviours. Two opposing stress-coping styles, categorized as proactive and reactive, can be identified. A 'proactive' stress-coping style is characterized by high levels of locomotor activity, aggression, and boldness, together with low HPI/A responsiveness and a high adrenergic response to a stressor (De Boer et al., 1990; Korte et al., 1992; Fokkema et al., 1995). Conversely, the 'reactive' stress-coping strategy is identified by the opposite spectrum of traits, including freezing and 'shy' behaviours (e.g. long latency to explore a novel object), as well as a higher cortisol response to stressors and a lower adrenergic response. One key difference between the two stress-coping styles is the degree of flexibility; proactive individuals tend to engage in routine behavioural patterns, whereas reactive individuals are more flexible and able to react to environmental stimuli, as the name suggests (Koolhaas et al., 1999). Which coping strategy is more adaptive depends on the environmental context, because proactive individuals thrive under stable conditions, but reactive individuals can maintain fitness in variable or unpredictable environments (Oortmerssen and Busser, 1989).

The present thesis explored the effects of maternal stress on offspring phenotype in larval fish. If the maternal environment is stressful because of variable or unpredictable conditions (e.g. populated with predators), then production of offspring with a more reactive stress-coping style would be predicted to be an adaptive maternal response, assuming the environment experienced by the offspring is matched to that of the mother. Koolhaas et al. (1999) argued that

tests assessing proactive 'choice' behaviours, such as measuring the latency to leave a refuge and explore a novel/aversive environment, are most discriminative in terms of categorizing an individual's stress-coping strategy as proactive or reactive (De Boer et al., 1990; Spoolder et al., 1996). The three anxiety tests described above each allow for the assessment of 'latency to explore' behaviours, thus providing the opportunity to assess the impact of maternal stress on offspring stress-coping style in multiple contexts.

1.8 Lab versus field experiments

The present thesis describes experiments conducted in both laboratory (Ch. 2, zebrafish) and field (Ch. 3, largemouth bass) settings. A laboratory approach is ideal for focused experiments exploring mechanisms, because the controlled environment allows the reduction of variation to better detect potential trends between treatment groups. Also, laboratory experiments usually can be repeated and/or troubleshooting can occur when technical issues arise. Conversely, time and/or resources are often limited in field studies (e.g. largemouth bass spawn once a year, Ostrand et al., 2004). To further complicate field work, survival often is difficult to measure in wild animal populations, owing to the difficulty in tracking fish over time. These issues are reflected in Table 1.1 by the lack of field studies assessing the effects of maternal stress on offspring survival in wild fish. Importantly for the present thesis, unless larval fish are housed in a laboratory setting, the survival of wild larval fish can only be estimated from the early stages of development when the offspring are located in a discrete area of the environment (i.e. nest).

On the other hand, it is important to conduct research that asks conservation-related questions on populations in the field, because laboratory animals are exposed to different types of stressors than wild animals (e.g. frequent handling vs predation, respectively) and thus, it is difficult to apply predictions from laboratory studies to how animals will react to a stimulus in the wild (Calisali and Bentley, 2009). Both basic and applied research increasingly are focused on the consequences of intensifying anthropogenic stressors (e.g. chemical pollution, climate change, invasive species, habitat degradation and fragmentation, etc.) to which wildlife are exposed (Wikelski & Cooke, 2006; Caro, 2007), and the endogenous production of GCs is arguably the most extensively studied response to such stressors (Cooke & O'Connor, 2010; Baker et al., 2013). Physiologically mimicking *in vivo* conditions of chronic stress experienced by wild species can generate predictions with mechanistic explanations as to how wildlife may cope with future stressors. From an applied perspective, data directly related to wild populations (not inferred to wildlife through experiments on laboratory species) can advise conservation managers and policy makers as to how susceptible certain species or populations are to anthropogenic stressors, and the ecological changes that responses to such stressors can elicit (Winder & Shindler, 2004). For this reason, the study described in Ch. 3 on intergenerational effects of cortisol injections in largemouth bass was conducted in the field.

Together, laboratory and field approaches allow conclusions to be drawn from controlled laboratory experiments that tease apart potential mechanisms behind observations made from field studies, and conversely, allow trends and phenomena observed in the laboratory to be tested on wild animals (reviewed by Costa and Sinervo, 2003; Calisali and Bentley, 2009).

1.9 Study species

The present thesis is comprised of two separate but related projects involving experiments conducted in the laboratory on zebrafish and in a natural field setting on largemouth bass. Both species are appropriate for intergenerational studies because they are oviparous animals that do not exhibit any maternal care to offspring. After fertilization, maternal stress during gestation can have long-term effects on offspring in viviparous (e.g. *Homo sapiens*; Brunton, 2013) and ovoviviparous (e.g. *Lacerta vivipara*; Uller and Olsson, 2006) species. Similarly, the hormonal status of oviparous females before and after fertilization can have long lasting effects on offspring when the female provides some form of maternal care to hatchlings (e.g. birds; reviewed by Monaghan and Haussmann, 2015). The present thesis was focused on the effects on offspring of maternal stress *prior* to fertilization, so oviparous species that do not provide maternal care provide an appropriate model.

Zebrafish are well-suited to intergenerational studies because they have a short generation time and adults can breed year round in the laboratory (Nasiadka and Clark, 2012). Additionally, females readily form dominance hierarchies (Jeffrey and Gilmour, 2016), providing a useful experimental paradigm with which to assess the effects of chronic maternal social stress associated with low social status. Furthermore, zebrafish are commonly used in laboratory experiments, so many physiological and behavioural techniques, including the measurement of the cortisol response to acute stressors (Jeffrey and Gilmour, 2016) and anxiety-related behavioural trials (Wilson et al., 2013; Maximino et al., 2010), already have been conducted and validated. In short, zebrafish proved to be a logistically simple species with which to conduct intergenerational studies.

Largemouth bass is an economically and culturally important fish species. As the most popular freshwater gamefish, modern bass fishing is a multibillion dollar industry worldwide (Williamson, 2016). Bass also are ecologically important for two reasons. First, bass prey upon the round goby (*Neogobius melanostomus*), which is invasive in the Great Lakes (Corkum et al., 2004), so largemouth bass act as a form of biocontrol. Second, largemouth bass have been introduced successfully into many countries, but their invasion has been associated with decline, displacement, and extinction of native species, mostly owing to predation (Kerr and Grant, 1999). For example, an endemic species of pupfish became extinct only one year after largemouth bass were introduced in Monkey Spring, Arizona (Minckley, 1973). Importantly for the purpose of the present thesis, a previous study by O'Connor et al. (2013) exposed wild largemouth bass females to exogenous cortisol prior to spawn and reported elevated ovarian cortisol concentrations several days post-injection (before and during spawn), thus providing the context to assess whether effects of this GC manipulation extend into the next generation.

1.10 Hypotheses and predictions

The present thesis tested the general hypothesis that maternal stress or maternal cortisol treatment (as a proxy for maternal stress) prior to spawn affects offspring anxiety behaviour and responsiveness to stress. Specifically, the present thesis assessed how maternal social stress affects zebrafish offspring and whether effects are hormonally-mediated via cortisol (Ch. 2), and explored whether experimentally elevated maternal cortisol has effects on the next generation in a field setting using wild largemouth bass (Ch. 3). Both projects built upon previous literature. Ch. 2 extended the work of Jeffrey and Gilmour (2016) on the effects of maternal social status on

development of the stress axis to older offspring and provided insight into how maternal social status affected offspring anxiety-related behaviour. Ch. 3 tested whether the observed effects in largemouth bass of maternal cortisol exposure prior to spawn, including elevated ovarian cortisol concentration documented by O'Connor et al. (2013), had an impact on the behaviour and physiology of the next generation.

In both studies, we predicted that offspring of treated females (i.e. females exposed to a social stressor or exogenous cortisol administration prior to spawn) would exhibit an attenuated cortisol response to an acute stressor and increased anxiety-related behaviour, including increased thigmotaxis and scototaxis, a longer latency to leave a refuge and explore a novel environment, lower levels of activity, and more freezing behaviour.

CHAPTER 2

EFFECTS OF MATERNAL EXPOSURE TO SOCIAL INTERACTIONS OR EXOGENOUS CORTISOL ON OFFSPRING SURVIVAL, CORTISOL LEVELS, AND BEHAVIOUR IN ZEBRAFISH (*Danio rerio*)

Note on Ch. 2

Ch. 2 is in preparation to be submitted to a peer-reviewed journal as:

JC Redfern, AE Brown, AR Shifman & KM Gilmour. Effects of maternal exposure to social interactions or exogenous cortisol on offspring survival, cortisol levels, and behaviour in zebrafish (*Danio rerio*).

PREFACE

Contributions of authors

JR, AEB, and KMG all contributed to concept development and experimental design. JR was responsible for data collection and analysis of the maternal social stress experiment. The maternal cortisol exposure experiment was carried out as part of the honours undergraduate thesis of AEB, which was mentored by JR, and is included in the present thesis for completeness because it will form part of the manuscript that will be submitted to a peer-reviewed journal. ARS wrote the Python script for the automatic tracking of larval zebrafish movement. JR composed the manuscript with the help of KMG.

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Ethics statement

All experiments were conducted at the University of Ottawa in compliance with the guidelines of the Canadian Council on Animal Care for the use of animals in research and teaching, and after approval of the University of Ottawa Animal Care Committee (protocol BL-2118).

2.1 ABSTRACT

Adult female zebrafish (*Danio rerio*) held in pairs interact to form a social hierarchy in which the subordinate female experiences chronic stress. The present study investigated whether maternal social stress affected offspring baseline and stress-induced cortisol levels, survival or anxiety-related behaviour. The extent to which effects of maternal social stress on offspring are mediated by maternal cortisol was tested by a parallel experiment in which female zebrafish were treated with exogenous cortisol prior to spawn. Offspring of dominant females exhibited greater boldness to explore novel areas as well as less geotaxic anxiety at 6 DPF. Offspring of control females had a similar tendency to exhibit greater boldness compared to offspring of cortisol-treated females. Embryos of cortisol-treated females did not have higher cortisol concentrations, suggesting the presence of a maternal buffering mechanism against embryonic hypercortisolism. Most strikingly, however, was the observation that females who had experienced social interaction, regardless of resulting social status, reared offspring that exhibited better survival at 1 DPF, a stronger freezing fear response at 6 DPF, and reduced baseline whole-body cortisol concentrations at 0 and 30 DPF compared to offspring of sham-treated females. The effects of maternal social interaction appeared to be independent of maternal cortisol levels, highlighting the need for further research to explore aspects of maternal stress beyond elevated maternal cortisol. Further investigation is required to determine whether the effects on offspring phenotype of acute maternal social stress associated with social interaction translate into improved offspring fitness in an environment matched to that of the mother in terms of exposure to stressors.

2.2 INTRODUCTION

Many vertebrates engage in antagonistic interactions when competing for limited resources such as food, territory, or mates, resulting in the formation of dominance hierarchies where the dominant individual gains preferential access to the limited resource (Creel 2001; McCormick, 1998; Sapolsky et al., 2000; reviewed by Love et al., 2013). In dyadic interactions, the "winner" becomes the dominant individual while the "loser" becomes the subordinate individual. During the period of hierarchy formation, both individuals typically experience acute stress, regardless of their resulting social status (Øverli et al., 1999; Thomas and Gilmour, 2006). For example, both rainbow trout (*Oncorhynchus mykiss*) within a pair exhibited increased concentrations of cortisol, the main GC stress hormone in teleosts (Wendelaar Bonga, 1997), after 5 min of interaction (Øverli et al., 1999). However, whereas cortisol concentrations returned to baseline levels within 3 hours in dominant fish, subordinate fish continued to exhibit sustained increases in circulating cortisol levels, characterizing the chronic social stress experienced as a result of their subordinate status (Sloman et al., 2001). Similar findings have been reported in zebrafish (*Danio rerio*), with cortisol levels of subordinate individuals being significantly higher than those of dominant individuals during dyadic interactions (Filby et al. 2010, Paull et al., 2010; Gronquist and Berges, 2013; Larson et al., 2006).

In general, chronic stress in teleosts has been associated with reduced growth, reproductive success and immune function (Paull et al., 2010; Pickering et al., 1982; Pickering and Pottinger, 1989; reviewed by Wendelaar Bonga, 1997). For example, chronic social stress associated with intraspecific competition resulted in reduced growth and condition factor in subordinate rainbow trout (Gilmour et al., 2005). Social stress in females prior to spawn may

affect not only the stressed individual, but effects also may extend to the next generation. For example, offspring of socially stressed female teleosts had poorer survival (Essington et al., 2000), increased cortisol content as embryos (McCormick, 1998, 2006), an attenuated cortisol response to an acute stressor (Jeffrey and Gilmour, 2016), and decreased area and length as embryos and larvae, respectively (McCormick, 1998, 2006, 2009). For female zebrafish confined in pairs for 2 days prior to fertilization (Jeffrey and Gilmour, 2016), reduced baseline cortisol concentrations were observed in 2 DPF offspring of dominant females (but no other difference in baseline cortisol up to 6 DPF was detected), together with an attenuated cortisol response in offspring at 6 DPF (but not at 4 DPF). These observations raise the question of whether effects of maternal social status on offspring HPI axis activity in zebrafish persist or change over time. Thus, the present study aimed to extend the work of Jeffrey and Gilmour (2016) by investigating the effects of maternal social status on offspring baseline and stress-induced cortisol levels up to 30 DPF. The cortisol response to stressors often is correlated with specific patterns of anxiety-related behaviour (reviewed by Koolhaas et al., 1999), and zebrafish larvae were reported to display "anxiety" as early as 4 DPF (Colwill and Creton, 2011). As such, the present study also explored whether maternal social status affected offspring activity, boldness, and anxiety-related behaviour using open-field, black-white preference, and novel tank diving tests (reviewed by Schnörr et al., 2012; Maximino et al., 2010; Blaser and Rosemberg, 2012).

Elevated maternal cortisol has been proposed as a possible mediator of maternal stress on offspring characteristics because cortisol (along with maternal mRNA, proteins, lipids, and hormones) is among the egg provisions deposited by pre-spawn females during vitellogenesis (Mousseau and Fox, 1998). Moreover, this GC is known to play an organizational role in early development in zebrafish, affecting embryonic development of the eye and HPI axis, the

formation of skeletal and cardiac muscle, and neurogenesis (Nesan and Vijayan, 2012, 2016). Previous research in zebrafish has reported that exposure of females prior to spawn to a fasting stressor or exogenous cortisol (via cortisol-laced food) resulted in increased maternal breeding success and fecundity (Best, 2015; Faught et al., 2016). Exposure of females or eggs to exogenous cortisol also increased offspring hatching success and thigmotaxic anxiety (Wilson et al., 2013), and caused morphological malformations (Nesan and Vijayan, 2012, 2016), a transient increase in embryo cortisol concentrations (Best, 2015; Faught et al., 2016), and an attenuated cortisol response to an acute stressor (Nesan and Vijayan, 2016). To tease out which effects of maternal social stress on offspring traits were mediated by maternal cortisol in the present study, pre-spawn female zebrafish were injected with exogenous cortisol and offspring characteristics were assessed, including offspring clutch size, survival, baseline and stress-induced cortisol, and anxiety-related behaviour.

Based on the previous studies in zebrafish including the work by Jeffrey and Gilmour (2016), it was predicted that socially subordinate (chronically stressed) females and females treated with exogenous cortisol prior to spawn would similarly have greater breeding success and fecundity, and would rear embryos with higher cortisol content that would hatch into larvae that exhibit greater survival, decreased larval baseline and stress-induced cortisol concentrations in response to an acute air exposure stressor, higher activity level, decreased boldness behaviour, and more anxiety-related behaviour.

2.3 MATERIALS AND METHODS

2.3.a Experimental animals

Adult zebrafish (*Danio rerio*) were either purchased from AQUAlity Tropical Fish Wholesale (Mississauga, ON) or obtained from in-house breeding at the University of Ottawa Aquatics Facility. Fish were housed in 3 or 10 L flow-through polycarbonate tanks under a 14 light:10 dark photoperiod, and tanks were supplied with aerated, dechloraminated city of Ottawa tap water ("system water") maintained at 28°C. Adult zebrafish were fed with No. 1 crumble-Zeigler (Aquatic Habitats, Apopka, FL, USA) to satiation three times daily and brine shrimp (*Artemia*) once daily. During the two day social interaction period (see below), adult females were fed crumbled food only.

From fertilization to 5 DPF, zebrafish embryos and larvae were held in 50 ml Petri dishes containing embryo medium (0.01% methylene blue, 0.275 g L^{-1} NaCl, 0.012 g L^{-1} KCl, 0.078 g L^{-1} $MgSO_4 \cdot 7H_2O$ and 0.046 g L^{-1} $CaCl_2 \cdot 2H_2O$) in a 28°C incubator, with a maximum density of 60 embryos or larvae per dish. Embryo medium was changed daily and dead embryos or larvae were removed. At 5 DPF, larvae were transferred to 1 L tanks containing dilute embryo medium (1:5 embryo medium:system water), with a maximum density of 50 larvae per L, and were fed processed crumbled food (ground adult crumbles) once daily. Larvae began receiving brine shrimp once daily at 16 DPF. Holding tanks were cleaned and dead larvae were removed every three days.

Upon experiment completion, all fish (embryos or larvae) were euthanized via terminal anaesthesia with buffered MS-222 (tricaine methanesulfonate; 0.72 mg ml^{-1} 3-aminobenzoic acid ethyl ester; 21 mM Tris, pH 7; Sigma-Aldrich, St. Louis, MO, USA; see Westerfield, 2000),

flash frozen in liquid nitrogen, and stored at -80^{o}C for later analysis of whole-body cortisol concentrations.

2.3.b Maternal treatments

Female zebrafish that had previously bred successfully were assigned to trials using social interactions or trials using exogenous cortisol treatment. Fish assigned to social interactions were matched with a conspecific based on similar mass and fork length (difference < 0.1 cm; n = 17 dominant and 18 subordinate individual females that produced offspring); fish that were handled in the same fashion but housed individually (sham; n = 16) served as the control. For cortisol-treated females (n = 18), fish that were neither handled nor treated served as the control (n = 18). Two days after the onset of maternal treatment, females were allowed to breed with size-matched males in individual breeding tanks.

2.3.b.i Exposure to social interaction

Females that had previously bred successfully were given a unique subdermal tattoo using alcian blue dye (A5268 Sigma-Aldrich). Fish were lightly anaesthetized with a buffered MS-222 solution (0.24 mg ml^{-1}) to the point of losing equilibrium, blotted dry, weighed, measured for fork length, and tattooed. After a 2 day recovery from tagging, size-matched fish were placed on their own as shams or in pairs on either side of an opaque, perforated divider within a social interaction arena (4.5 L). The next morning, the divider was removed, and the paired females were allowed to interact. Fish were observed for 3 min twice daily for two days (4 observations total) and social status was assigned based on a points system that assessed each female's position within the water column, feeding (first fish to eat a single food pellet),

aggression (chases and charges), and submissive behaviours such as retreats and freezing (see Appendix B). Acts of dominance, such as patrolling the middle of the water column, monopolizing food and chasing, received higher scores than subordinate behaviours such as freezing and retreating in response to being chased. The individual with the higher overall score within the pair was assigned dominant status. Similar scoring systems have previously been used to assess social dominance in zebrafish (Filby et al., 2010; Paull et al. 2010) as well as other teleosts (rainbow trout, *Oncorhynchus mykiss*, Jeffrey et al., 2012 and 2014). Pairs for which the difference in behaviour score was not statistically significant (paired Student's *t*-test, $P > 0.05$, n = 4 observations) were excluded from the remainder of the experiment (3 pairs excluded; 9% of pairings).

2.3.b.ii Exposure to exogenous cortisol

Cortisol-treated females were anaesthetized in a buffered solution of MS-222 (0.24 mg ml^{-1}), and injected intramuscularly with 3 µl of 18 ng μl^{-1} hydrocortisone 21-hemisuccinate (H4881 Sigma-Aldrich) in 0.9% NaCl. This dose was chosen on the basis of a previous study where it elevated whole-body cortisol levels to 27.3 ± 4.5 ng g^{-1} (A. Castle, unpublished observations), a level comparable to documented elevations in whole-body cortisol concentrations in adult zebrafish following exposure to an acute netting stressor (~28 ng g^{-1}; Ramsay et al., 2009). In previous studies on other fish species, fish treated with the injection vehicle alone (i.e. saline or cocoa butter) exhibited circulating cortisol concentrations that were intermediate between minimally-handled (control or untreated) and cortisol-treated teleosts (McCormick, 1998; DiBattista et al., 2005; O'Connor et al., 2009; 2010; Dey et al., 2010), complicating interpretation of the effects of elevated cortisol levels. For this reason, and to ensure the largest

possible difference between treatments in terms of cortisol levels, a true control rather than a sham-treated group was included in the experimental design.

2.3.c Breeding and offspring acquisition

Males that had previously bred successfully were sorted by fork length and mass into three size groups (n = 12 for each size group). Females of all treatments within one experimental trial were bred with males from the same group to standardize for effects of paternal size. Although *in vitro* fertilization was the initial method of choice for breeding because it allowed for the pooling of milt from several males (Jeffrey and Gilmour, 2016), this method resulted in very poor fertilization success (<20%) in the present study. As such, natural breeding as per Nasiadka and Clark (2012) was instead adopted (> 50% breeding success). The transparent divider was removed the following morning when the lights were turned on, allowing the fish to breed. Breeding success, defined as the percentage of females within a maternal treatment group that produced a viable clutch of embryos, was recorded.

From the females that bred successfully, all eggs and embryos were collected from breeding tanks, bathed in a dilute bleach solution (3 ml 0.5% sodium hypochlorite in 200 ml of dechloraminated system water) for 2 min, and then placed in Petri dishes containing embryo media (as outlined in 2.3.a). Offspring were raised to 30 DPF, during which time survival, baseline and stress-induced cortisol concentrations, and anxiety-related behaviours were assessed.

2.3.d Assessment of offspring characteristics

2.3.d.i Survival

All eggs/embryos were counted to determine total clutch size upon collection from individual breeding tanks. Non-viable, opaque eggs/embryos were counted and removed to determine the percentage of viable embryos within each female's clutch. Survival of viable offspring was re-assessed at 1 DPF, 2 DPF (time of hatching), 4 DPF, and 6 DPF as per Jeffrey (2014).

2.3.d.ii Baseline and stress-induced cortisol concentrations

Offspring were raised to 6, 15, and 30 DPF. At each time, groups of 14-50 larvae (depending on offspring size) were euthanized (0.72 mg ml^{-1} buffered MS-222) and flash frozen in liquid nitrogen either immediately (baseline cortisol levels) or following 1 min air exposure in a handheld net and 5 min of recovery in system water (stress-induced cortisol levels). The 1 min air-exposure stressor was chosen based on preliminary trials in which air exposure (compared to swirling, high or low temperature, or an osmotic stressor) most consistently elicited a cortisol response across different ages, 6-30 DPF, of larvae (data not shown). A 5 min recovery period was chosen because stress-induced cortisol levels peak and then fall after 5 min of recovery post-air exposure in zebrafish larvae (A. Hare, unpublished observations). The stress response of embryos was not tested because zebrafish do not mount a cortisol response to external stressors prior to hatch (Alsop and Vijayan, 2008). Samples were stored at -80^{o}C until later analysis of whole-body cortisol concentrations.

Cortisol was extracted from pooled larvae as per Jeffrey and Gilmour (2016) and quantified by enzyme-linked immunosorbent assay (EIA). In brief, thawed samples were homogenized on ice in 200 µL of 5X diluted extraction buffer from a commercial cortisol EIA kit (Cortisol EIA assay kit, #402710 Neogen Corporation—Life Sciences, Lexington, KY, USA) using a battery operated pellet pestle (BioMasher II®, Kimble Chase Kontes, Vineland, NJ, USA). Homogenates were extracted thrice into 1 ml ether (#AC615080010 anhydrous diethyl ether, Fisher Scientific, Pittsburgh, PA, USA) each time. After each addition of ether, samples were vortexed thoroughly, incubated at room temperature for 15 min (30 min the first time), centrifuged for 5 min at 3,000 *g* at 4°C, and flash frozen at -80°C for 30 min. The liquid phases of each extract were decanted, combined and dried under forced air at room temperature. The residue was reconstituted in 10 µL extraction buffer (Neogen) per larva in the sample. To aid reconstitution, samples were heated for five min at 65°C and vortexed at least twice or until the residue was completed dissolved. Extraction efficiency, which was determined by spiking homogenates with a known amount of radio-labelled cortisol (^{3}H-hydrocortisone, 250 µCi, #NET396250UC Perkin Elmer, Waltham, MA, USA), was 77 ± 18%. Extracts were stored at -80°C until quantification of cortisol content (Neogen). Samples were assayed in duplicate. Inter- and intra-assay variabilities were 5.8% and 2.5%, respectively.

2.3.d.iii Behaviour:

Subsets of 5 larvae from each brood were randomly selected to perform an open-field test (6 DPF; only for offspring of paired and sham females), a novel tank diving test (6 DPF; for offspring of all experimental females), or a black-white preference test (7 DPF; for offspring of all experimental females) to assess anxiety-related behaviour. Fish were recorded (Canon Vixia

HF-R400 camcorder) at 30 frames per second (fps) individually in an isolated room. Aside from the wall of the testing arena through which behaviour was recorded, the walls of all testing chambers were opaque to eliminate disturbances from movement outside the testing arena. Also, with the exception of the novel tank diving test, which required the entire behavioural arena to be "novel" to the test subject, individual fish were allowed to acclimate prior to recording for 2 min (Toms and Echevarria, 2014) in a starting chamber (PVC pipe, 3 cm diameter x 6 cm high) to minimize confounding behavioural responses associated with handling. Larvae were tested individually and were subjected to only a single behaviour test (and one trial) to avoid habituation or stress associated with repeated handling. Lastly, to avoid effects of temperature changes on behaviour, water was changed after 5 offspring from the same clutch were individually recorded (i.e. before testing offspring from another female's clutch). Because any variation in water temperature was equal among all maternal treatment groups, effects due to the slight drop in water temperature that occurred during testing (maximum 28 ^{o}C to 26 ^{o}C) also would have been consistent across all maternal treatment groups.

Open-field test: This commonly-used test assessing thigmotaxic anxiety (i.e. "wall-hugging", Schnörr et al., 2012; Nishio et al., 2001) and activity (Burns, 2008; Toms and Echevarria, 2014) was conducted in an opaque, white behavioural arena to facilitate visualization of the 6 DPF zebrafish larvae. The testing chamber consisted of a 10 cm diameter plastic container filled with 3 cm system water. After a 2 min acclimation period, the starting chamber was removed and the test subject was recorded for 5 min. Movement of a larva in each recording was tracked automatically using a script written in Python. The steps involved in extracting behavioural endpoints from the resulting x and y coordinates (output from Python script) are described in Appendix C. Offspring thigmotaxic anxiety was assessed by quantifying

the percentage of time an individual spent in the outer third of the test arena, a measure of the anxiety-related behaviour of thigmotaxis. General activity level was also assessed by measuring the percentage of time larvae spent actively swimming (immobility defined as less than 1 mm of movement within $1/30^{th}$ s; i.e. within one video frame), the frequency of freezing bouts (number of bouts per min; one bout defined as being immobile for at least 2 s), the total distance travelled while actively swimming during the test (cm), and the average swimming velocity.

Novel tank diving test: This test assessed geotaxis, an anxiety-related behaviour characterized by the tendency of a fish to seek protection in a novel environment by diving down until it becomes "bold enough" to explore the remainder of the exposed water column (Levin et al., 2007; Sackerman et al., 2010; Maximino et al., 2010; Egan et al., 2009). This test is conceptually similar to the open-field test, but assesses vertical movement whereas the open-field test quantifies horizontal movement. In the present study, the movement of individually tested larvae was recorded for 5 min. The behaviour chamber (12 x 6.5 cm at the top, 10 x 5 cm at the bottom, 8 cm tall) contained approximately 200 ml of system water (6 cm deep) and was bordered on three sides by white opaque walls, while the front-facing wall was clear to allow visualization and video recording of the test subject's vertical movement. The tank was subdivided into three areas by depth, each 2 cm deep. The area in which the larva immediately entered upon being transferred to the testing chamber was noted as the "home area" (typically the bottom third of the tank, occasionally the top third of the tank, never the middle). The remaining two areas were then deemed to be "novel areas" for that particular individual trial. The percentage of time offspring spent in the home area (i.e. geotaxic anxiety) and the latency to enter the novel area in the middle of the water column (inverse measure of boldness) were

quantified from video recordings by an observer who was blind to the maternal treatment group to which an individual larva belonged.

Black-white preference test: This test assessed the anxiety-related behaviour of scototaxis, which is defined by an individual's preference for a dark environment when anxious (Steenbergen et al., 2011; Maximino et al., 2010; 2011). One half of the round testing chamber was painted black and the other half, white. The starting chamber was removed after 2 min of acclimation and the behaviour of the test subject was subsequently recorded for 5 min. To assess scototaxis, the percentage of time spent in the black half of the tank was quantified from the video recordings by eye. Again, to avoid bias while scoring videos, the observer was blind to the treatment group to which the offspring belonged.

2.3.e Statistical analyses

Offspring traits were compared among dominant, subordinate, and sham-treated females, and separately between control and cortisol-treated females, reflecting the fact that data were collected in two separate experiments. All data were expressed as mean values ± 1 standard error of the mean (SEM). All data analysis was conducted using R (version R i386 3.1.1; R Core Team, 2013). The assumptions of normally distributed residuals and homoscedasticity were first tested for each measured trait in R using the Shapiro-Wilk Normality Test (Royston, 1995) and Bartlett's Test of Homogeneity of Variances (Bartlett, 1937). Where significant deviation from normal distribution of residuals was detected, the data were transformed using the ladder of powers (Velleman and Hoaglin, 1981) according to Kirchner (2001), in which case the minimum appropriate transformation required to achieve normality of the residuals was chosen. The fixed

effect of maternal treatment (social status or cortisol treatment) was assessed for each endpoint using a one-way analysis of variance (ANOVA) or Student's *t*-test, respectively, except for cortisol concentrations, where the effects of both maternal treatment and air stressor exposure were evaluated in a two-way ANOVA (assessing effect of maternal treatment and air exposure). Where additional variables had to be taken into account, such as maternal ID or date/experiment number, the Akaike information criteria (AIC) of at least two models were compared: a general linear model testing only the fixed effect of maternal status/treatment on the response variable and at least one linear mixed effects model (nlme R package, Pinheiro et al., 2015) that incorporated any possible random effect(s) such as maternal ID or experiment number/date. Continuous variables that were potential sources of error were also taken into account as a second independent variable added to the base model (e.g. the potential effect of the number of larvae within a tube when measuring average larval cortisol concentration). If there was more than one applicable random effect, then multiple mixed effects models were created in stepwise order. That is, variations of the base model plus each individual random variable separately were created, and then (an) additional model(s) combining the random effects were also included in the AIC model comparison. Candidate models were objectively compared using the Restricted Maximum Likelihood estimation to find the optimal random structure. The model with the lowest AIC value was deemed the best fit and was used to conduct a parametric test (ANOVA for maternal social status and Student's *t*-test for maternal cortisol treatment). A significance value $\alpha = 0.05$ was used for all statistical tests.

2.4 RESULTS

Social status did not have a significant effect on the likelihood of a female to breed successfully (80%, 59%, and 62% for sham, dominant, and subordinate females, respectively; Likelihood ratio chi-square test; P = 0.146); however, it should be noted that females that underwent dyadic social interactions to form a social hierarchy exhibited marginally poorer breeding success compared to sham females. Of the females that successfully bred, the size of the clutch produced (i.e. fecundity) was not influenced by the female's social status (171 ± 26, 192 ± 32, and 226 ± 36 eggs per clutch for sham, dominant, and subordinate females, respectively; ANOVA, linear mixed effects model, square root transformed data with experiment date as a random effect; P = 0.813). However, a tendency for clutches from subordinate females to have a larger percentage of viable embryos compared to sham females was detected (Fig. 2.1.A; ANOVA, arcsine square root power transformed data; P = 0.069). From the experiment on cortisol-treated females, no effects of maternal cortisol treatment on clutch size (108 ± 19 eggs for control and 114 ± 21 eggs for cortisol-treated females; Student's *t*-test; P = 0.842) or the percentage of viable embryos within a clutch were detected (77.3 ± 7.7% and 77.3 ± 7.6% clutch viability for control vs cortisol-treated females; n = 18, Student's *t*-test; P = 0.997).

Offspring survival was measured at 1, 2, 4, and 6 DPF. At 1 DPF, offspring of dominant and subordinate females exhibited significantly greater survival than offspring of sham females (Fig. 2.1.B; ANOVA, linear mixed effects model, arcsine power transformed data with experiment date as a random effect; P = 0.004), with survival of 96.1 ± 1.3% (n = 33) for pooled offspring of dominant and subordinate females versus 78.9 ± 4.6% (n = 16) for offspring of sham females. Similarly, a trend for embryos of dominant and subordinate females to exhibit lower

cortisol content than those reared from sham females was detected (1.20 ± 0.49, 0.317 ± 0.044, and 0.570 ± 0.184 pg per embryo reared from sham, dominant, and subordinate females, respectively; ANOVA; n = 7-10; P = 0.096). Indeed, pooling offspring of dominant and subordinate females revealed that maternal social interaction was associated with significantly reduced offspring cortisol content right after spawn (Fig. 2.1.C; Student's *t*-test; P = 0.038). All effects of social interaction on offspring survival dissipated after 1 DPF. Maternal cortisol treatment did not have an effect on offspring survival at 1 DPF (76.5 ± 7.7% and 76.0 ± 7.6% survival for offspring of control and cortisol-treated females, respectively; n = 18; Student's *t*-test; P = 0.964) or any of the other three ages assessed up to 6 DPF (data not shown; Student's *t*-test at each age, P > 0.05).

The cortisol response to an acute air stressor was assessed at 6, 15, and 30 DPF. At all ages tested, the mean stress-induced whole-body cortisol concentration was significantly higher than the mean baseline whole-body cortisol concentration, thus validating the use of 1 min of air exposure as the acute stressor. Maternal social status did not have a significant effect on whole-body cortisol concentrations in larval offspring at 6 DPF (Fig. 2.2.A; two-way ANOVA, 5^{th} root transformed data; P = 0.380 for maternal social status, P = 0.021 for air, P = 0.782 for status x air), 15 DPF (Fig. 2.2.B; two-way ANOVA; P = 0.825 for status, P = 0.028 for air, P = 0.845 for status x air), or 30 DPF (Fig. 2.2.C; two-way ANOVA, 7-root transformed data; P = 0.067 for status, P = 0.002 for air, P = 0.576 for status x air). The trend in baseline whole-body cortisol at 30 DPF coupled with the P-value of 0.067 for maternal status (Fig. 2.2.C) suggested that further investigation could be worthwhile. Indeed, offspring of dominant and subordinate females tended to produce offspring with lower baseline cortisol at 30 DPF compared to offspring of sham females (Fig. 2.2.C; ANOVA, cube root transformed data; n=8-10; P = 0.093). Again,

pooling baseline whole-body cortisol concentrations for 30 DPF offspring of dominant and subordinate females together into a single "social interaction" treatment revealed an overall effect of maternal social interaction on baseline cortisol levels, with offspring of mothers that had engaged in social interactions having significantly lower baseline whole-body cortisol content at 30 DPF than offspring of sham females (Fig. 2.3.A; Student's *t*-test, cube root transformed data; $P = 0.029$). There was no significant effect of maternal cortisol treatment on offspring baseline whole-body cortisol concentrations (Fig. 2.3.B; Student's *t*-test; $P = 0.595$) at 30 DPF. There was also no significant effect of maternal cortisol treatment (MT) on mean offspring baseline or stress-induced whole-body cortisol concentration at the earlier ages tested (6 DPF, Fig. 2.4.A, two-way ANOVA, square root transformed data, $P = 0.609$ for MT, $P = 0.001$ for air, $P = 0.226$ for MT x air; 15 DPF, Fig. 2.4.B, two-way ANOVA, reciprocal transformed data, $P = 0.916$ for MT, $P < 0.001$ for air, $P = 0.730$ for MT X air; 30 DPF, Fig. 2.4.C, two-way ANOVA, log transformed data, $P = 0.399$ for MT, $P < 0.001$ for air, $P = 0.228$ for MT X air).

The anxiety-related behaviour of thigmotaxis (% time spent in outermost third of the open field test chamber) in 6 DPF larval offspring did not differ among maternal treatments (Fig. 2.5.A; ANOVA, linear model using generalized least squares; $P = 0.825$). Maternal social experience rather than maternal social status affected activity level (i.e. distance travelled, velocity, and percentage of time spent active) in the open-field test. Specifically, offspring of sham, dominant, and subordinate females did not significantly differ in their average swimming velocity (4.44 ± 0.19 cm s^{-1}, 5.44 ± 0.42 cm s^{-1}, and 5.11 ± 0.35 cm s^{-1} for offspring of sham, dominant, and subordinate females, respectively; ANOVA, reciprocal transformed data; $n = 15$-23; $P = 0.307$) or total distance travelled (23.4 ± 6.8 cm, 14.6 ± 3.3 cm, and 10.1 ± 1.7 cm for

offspring of sham, dominant, and subordinate females, respectively; ANOVA, quad root transformed data; n = 16-23; P = 0.206). Although the resulting status did not affect total distance travelled, a trend was revealed for offspring of females that engaged in dyadic social interactions (dominant and subordinate females) to swim a shorter total distance compared to offspring of sham females (Fig. 2.5.B; Student's *t*-test, quad root transformed data; P = 0.090), likely owing to the observation that offspring of dominant and subordinate females also tended to engage in half as much active swimming compared to offspring of sham females (1.4 ± 0.3%, 0.7 ± 0.1%, and 0.6 ± 0.1% for offspring of sham, dominant, and subordinate females, respectively; ANOVA, power transformed data; n = 16-22; P = 0.063). Thus, offspring of females that engaged in social interactions exhibited significantly less time spent actively swimming compared to offspring of sham females (Fig. 2.5.C; Student's *t*-test, power transformed data; P < 0.001). This effect of maternal social experience on offspring activity level was not explained by the number of times that offspring "froze", where a bout of freezing was defined as at least 2 seconds (s) of immobility, because offspring of dominant and subordinate females did not freeze more often than offspring of sham females (5.7 ± 0.7, 4.5 ± 0.4, and 4.4 ± 0.4 freezing bouts per min exhibited by offspring of sham, dominant, and subordinate females, respectively; ANOVA, linear mixed effects model with experiment date as a random effect; n = 17-23; P = 0.280). Put differently, offspring of females that engaged in dyadic interactions did not stop swimming more often; instead, larvae remained immobile for longer periods of time while "freezing" compared to offspring of sham females, thus resulting in less time spent active and a shorter overall distance covered.

The novel tank diving test revealed effects of maternal social status, maternal dominance in particular, on geotaxic anxiety and boldness in 6 DPF larvae. Specifically, offspring of

dominant females spent a smaller percentage of time in the home area, thus exhibiting less geotaxic anxiety, than offspring of sham and subordinate females (Fig. 2.6.A; ANOVA, arcsine transformed data; $P = 0.009$). This effect of maternal status was not explained by maternal cortisol because maternal treatment with exogenous cortisol did not affect offspring geotaxic anxiety (Fig. 2.6.B; Student's *t*-test; $P = 0.121$). Along with an increased percentage of time spent in novel areas of the tank, offspring of dominant females also exhibited greater boldness characterized by a shorter latency to exit the home area and explore the novel areas compared to offspring of sham and subordinate females (Fig. 2.6.C; ANOVA, quad root transformed data; $P < 0.001$). This observation of a quicker tendency to explore in offspring of dominant females may be linked at least in part to maternal cortisol because offspring of control females tended to similarly explore sooner than offspring of cortisol-treated females (Fig. 2.6.D; Student's *t*-test, quad root transformed data; $P = 0.098$).

No effect of maternal social status on offspring scototaxic anxiety behaviour was detected by the black-white preference test. That is, the percentage of time 7 DPF offspring of sham, dominant, and subordinate females spent in the black half of the behaviour arena (i.e. exhibiting scototaxis) did not differ significantly with maternal status (Fig. 2.7.A; ANOVA, linear mixed effects model with maternal ID as a random effect; $P = 0.458$). Maternal cortisol treatment also did not have an effect on the scototaxic anxiety exhibited by 7 DPF larvae (Fig. 2.7.B; Student's *t*-test; $P = 0.951$).

Figure 2.1 Characteristics of offspring of sham, dominant, and subordinate female zebrafish (*Danio rerio*) within 1 day of spawning. The percent survival of offspring at 0 (i.e. percent clutch viability; A; n = 11-15) and 1 day-post-fertilization (DPF; B; n = 16-17), as well as embryo whole-body cortisol concentrations (picograms, pg; 0 DPF; C; n = 7 sham and 18 interacting females) are presented as means + SEM. Groups that do not share a letter are significantly different from one another (Student's *t*-test and ANOVA; see text for details).

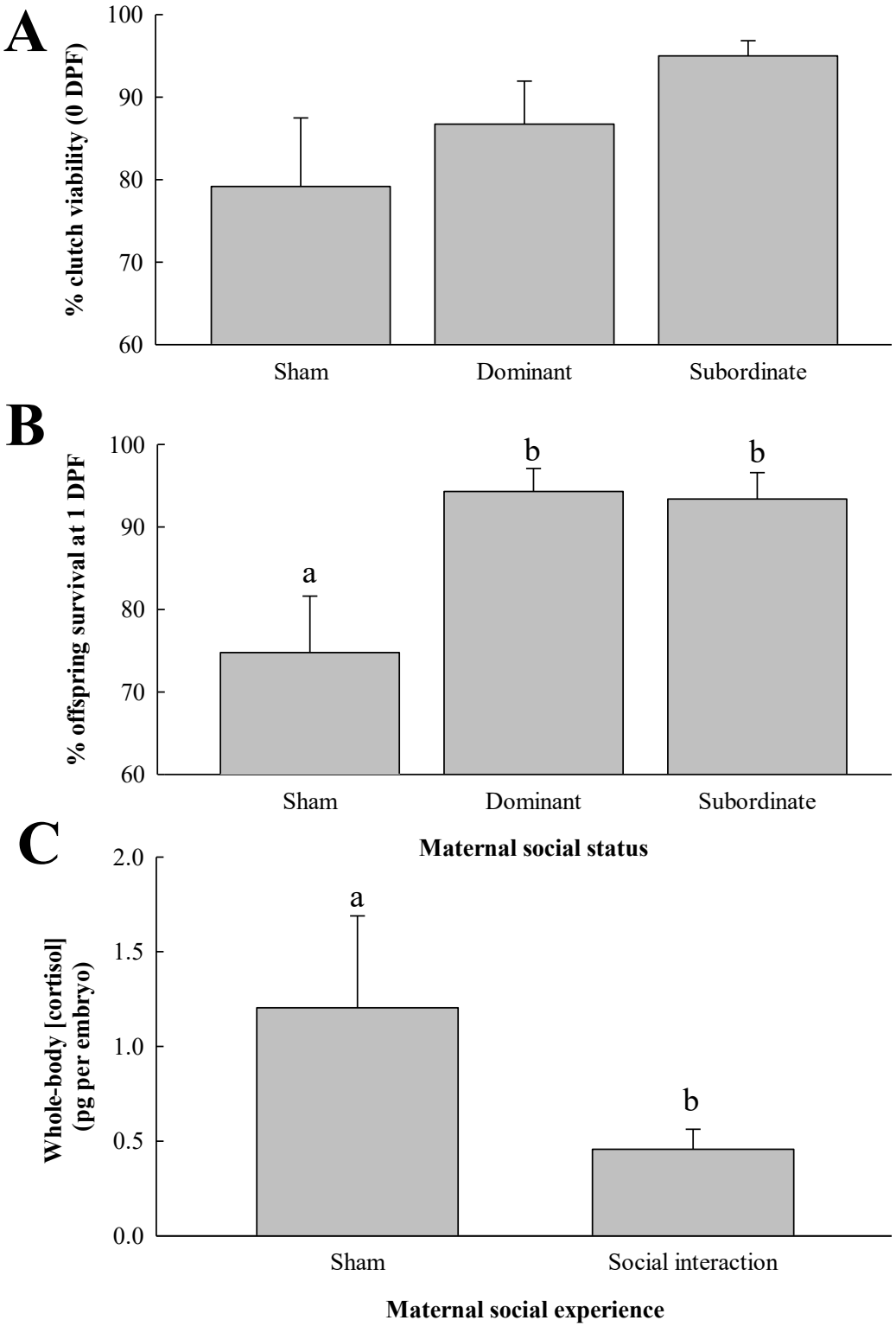

A
% clutch viability (0 DPF)
100
90
80
70
60
Sham
Dominant
Subordinate
B
% offspring survival at 1 DPF
100
90
80
70
60
a
b
b
Sham
Dominant
Subordinate
Maternal social status
C
Whole-body [cortisol] (pg per embryo)
2.0
1.5
1.0
0.5
0.0
a
b
Sham
Social interaction
Maternal social experience

Figure 2.1

Figure 2.2 Effects of maternal social status on offspring baseline and stress-induced whole-body cortisol concentrations (picograms, pg) in zebrafish (*Danio rerio*) at 6 (A; n = 7-12), 15 (B; n = 6-8), and 30 DPF (C; n = 8-10). Values are means + SEM. Stress-induced cortisol levels were measured following a 1 min acute air exposure stressor and 5 min recovery in water. Groups that do not share a letter are significantly different from one another (two-way ANOVA; see text for details); where no letters are present, no statistically significant differences were detected.

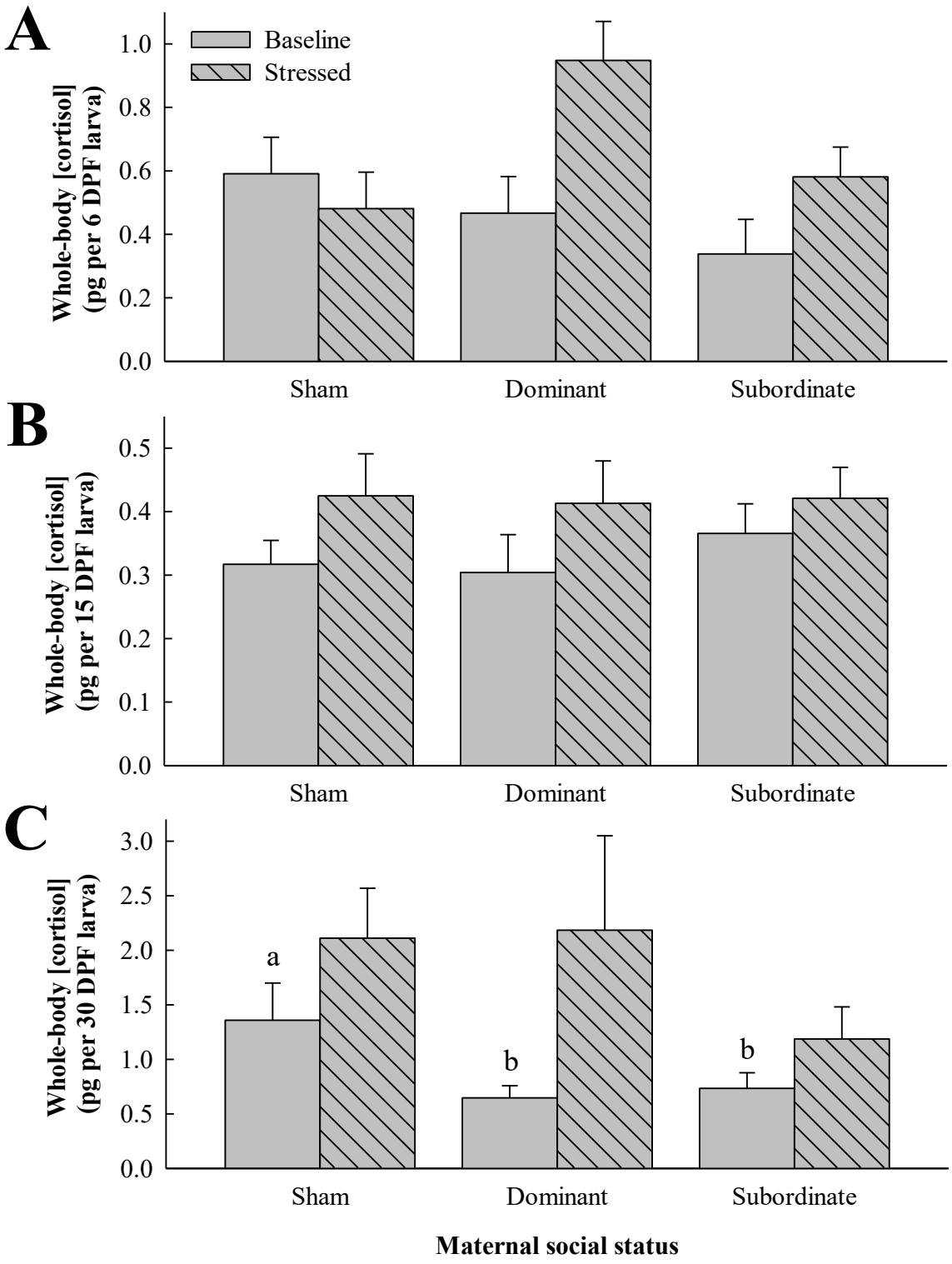

A
Baseline
Stressed
Whole-body [cortisol]
(pg per 6 DPF larva)
1.0
0.8
0.6
0.4
0.2
0.0
Sham
Dominant
Subordinate
B
Whole-body [cortisol]
(pg per 15 DPF larva)
0.5
0.4
0.3
0.2
0.1
0.0
Sham
Dominant
Subordinate
C
Whole-body [cortisol]
(pg per 30 DPF larva)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
Sham
Dominant
Subordinate
Maternal social status

Figure 2.2

Figure 2.3 Effects of maternal exposure to social interactions (A; n = 8 for sham and 20 for social interaction) and exogenous cortisol (B; n = 8 for control and 10 for cortisol-treated) on baseline whole-body cortisol concentrations (pg, picograms) in 30 DPF zebrafish (*Danio rerio*) larvae. Values are presented as means + SEM. Groups that do not share a letter are significantly different from one another (Student's *t*-test; see text for details); where no letters are present, no statistically significant differences were detected.

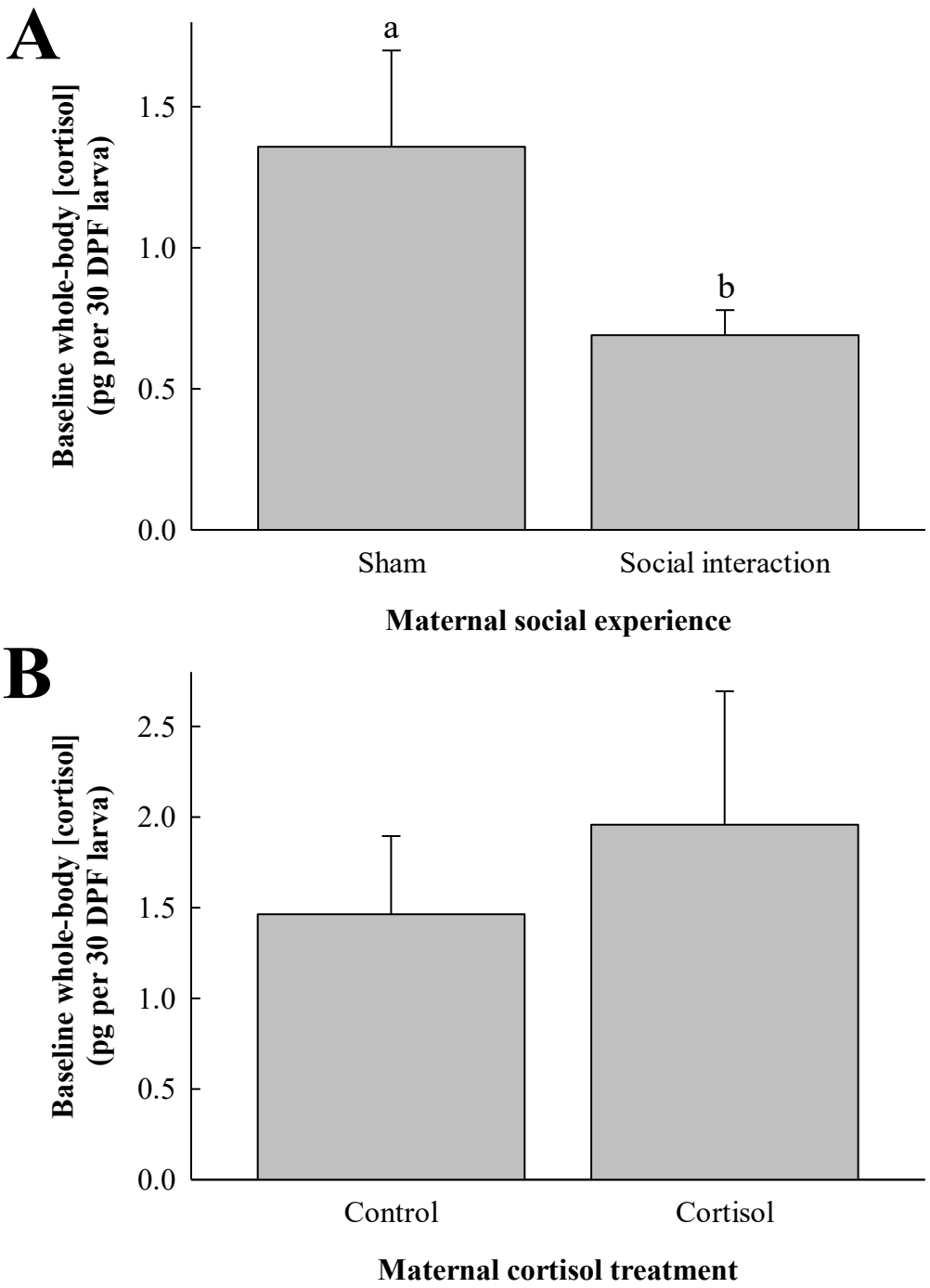

A
a
b
1.5
1.0
0.5
0.0
Baseline whole-body [cortisol]
(pg per 30 DPF larva)
Sham
Social interaction
Maternal social experience
B
2.5
2.0
1.5
1.0
0.5
0.0
Baseline whole-body [cortisol]
(pg per 30 DPF larva)
Control
Cortisol
Maternal cortisol treatment

Figure 2.3

Figure 2.4 Effects of maternal cortisol treatment on offspring baseline and stress-induced whole-body cortisol concentrations (picograms, pg) in zebrafish (*Danio rerio*) larvae at 6 (A; n = 6-8), 15 (B; n = 7-8), and 30 DPF (C; n = 8-10). Stress-induced cortisol levels were measured following a 1 min acute air exposure stressor and 5 min of recovery in water. Values are means + SEM; no statistically significant effects of maternal cortisol treatment were detected (two-way ANOVA; see text for details).

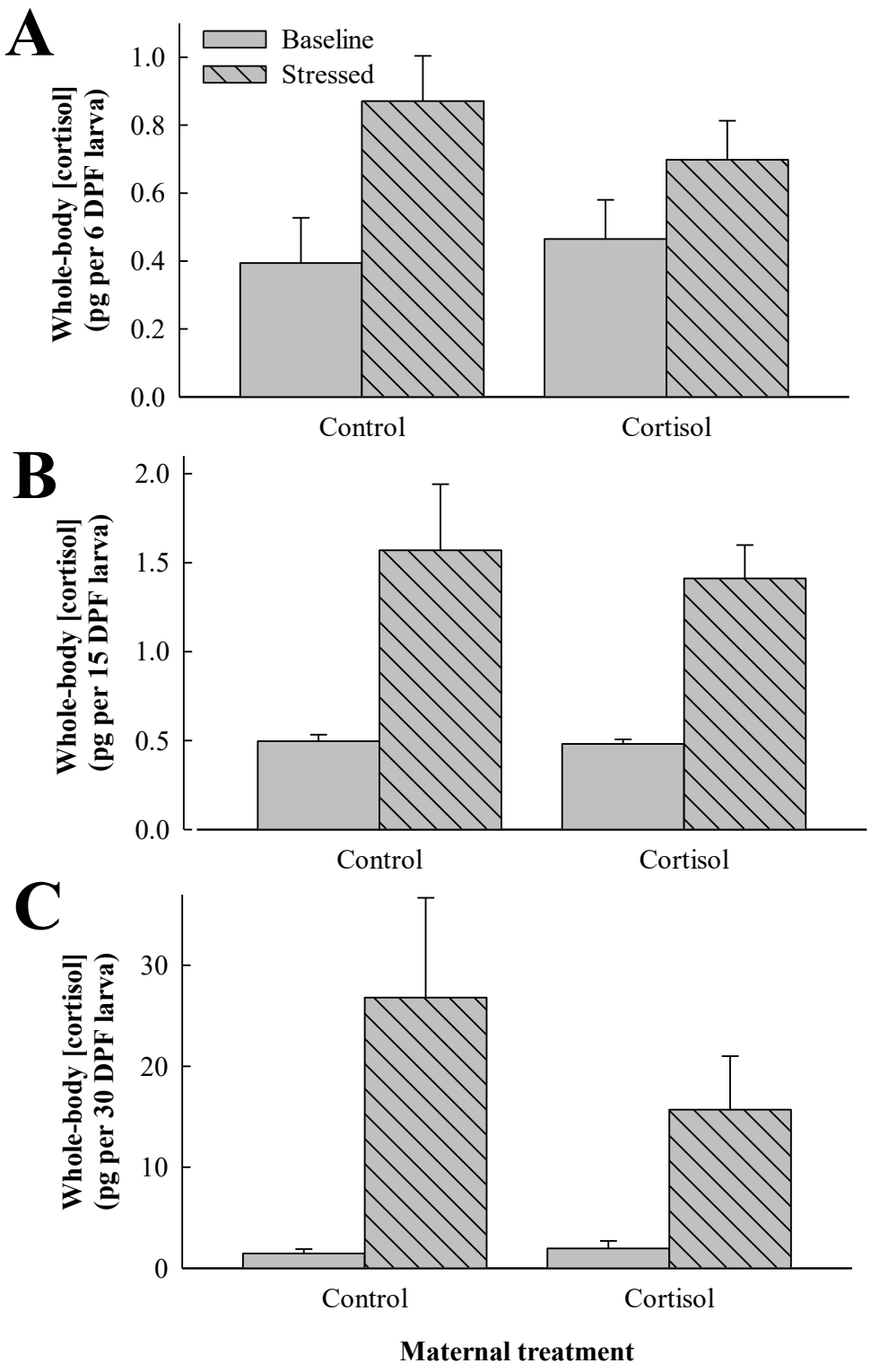

A
Baseline
Stressed
Whole-body [cortisol]
(pg per 6 DPF larva)
1.0
0.8
0.6
0.4
0.2
0.0
Control
Cortisol
B
Whole-body [cortisol]
(pg per 15 DPF larva)
2.0
1.5
1.0
0.5
0.0
Control
Cortisol
C
Whole-body [cortisol]
(pg per 30 DPF larva)
30
20
10
0
Control
Cortisol
Maternal treatment

Figure 2.4

Figure 2.5 Effects of maternal social status on the behaviour of 6 DPF zebrafish (*Danio rerio*) offspring in an open-field test. The percentage of time offspring of sham (n = 15-16), dominant (n = 22-23), and subordinate (n = 19-21) females spent in the outer area of the behaviour arena (thigmotaxic anxiety; A), as well as the total distance travelled by individual offspring (B) and the percentage of time offspring of sham females (n = 15-16) and females exposed to dyadic social interactions (n = 43) spent engaged in active swimming (C) during the 5 min test are illustrated. Values are means + SEM. Groups that do not share a letter are significantly different from one another (ANOVA and Student's *t*-test for assessing effects of maternal status and social experience, respectively; see text for details); where no letters are present, no statistically significant differences were detected..

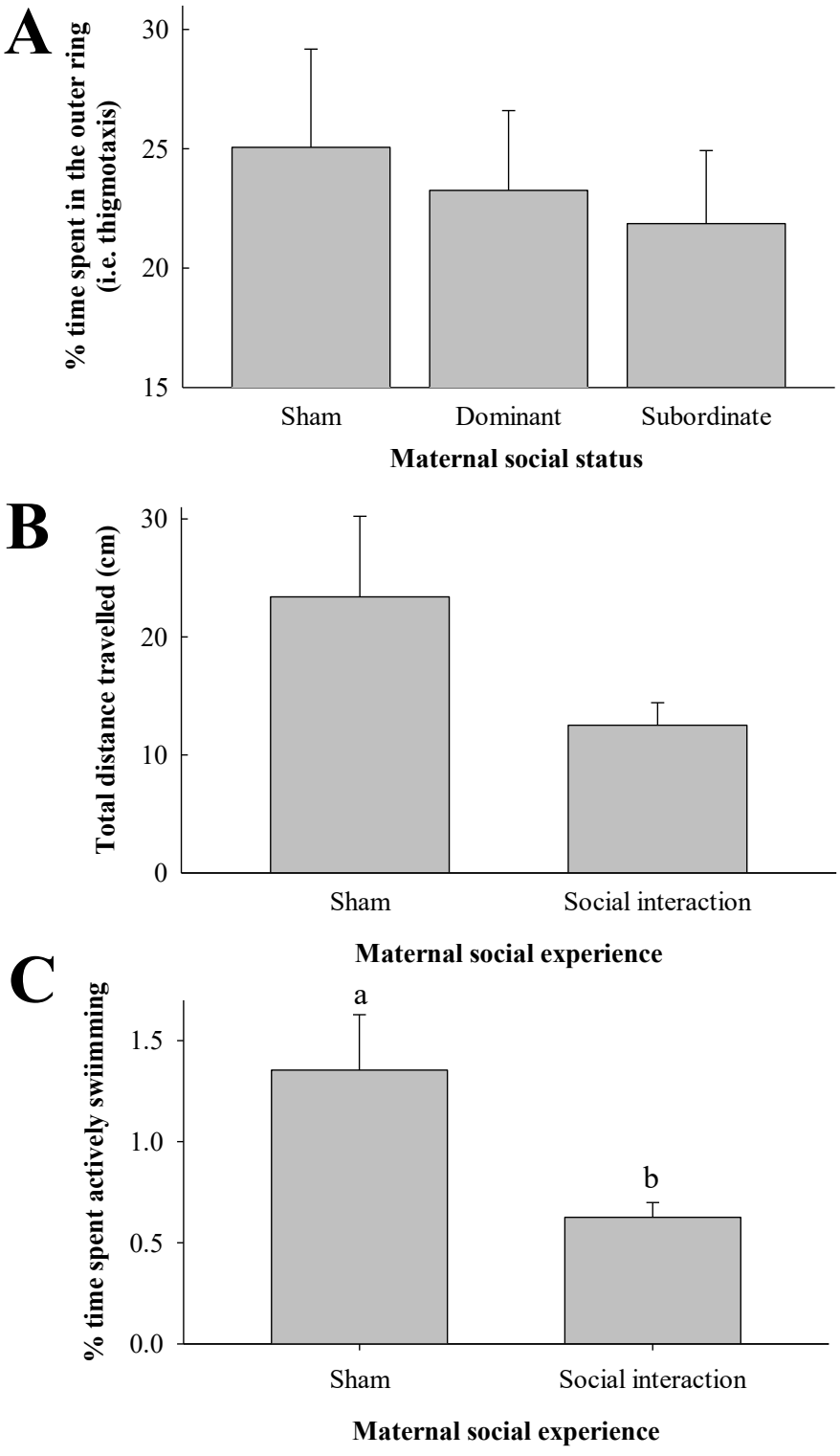

A
% time spent in the outer ring (i.e. thigmotaxis)
30
25
20
15
Sham
Dominant
Subordinate
Maternal social status
B
Total distance travelled (cm)
30
20
10
0
Sham
Social interaction
Maternal social experience
C
% time spent actively swiimming
a
b
1.5
1.0
0.5
0.0
Sham
Social interaction
Maternal social experience

Figure 2.5

Figure 2.6 Effects of maternal social status and cortisol treatment on the percentage of time 6 DPF zebrafish (*Danio rerio*) offspring spent in the home refuge area (exhibiting geotaxic anxiety; A—status; B—cortisol treatment; n = 46, 42, 58, 17, 16 offspring of sham, dominant, subordinate, control, and cortisol-treated females, respectively) and their latency to leave the home area and explore novel areas (C—status; D—cortisol treatment; n = 45, 39, 57, 18, 15 offspring of sham, dominant, subordinate, control, and cortisol-treated females, respectively) during the novel tank diving test. Values are presented as means + SEM. Groups that do not share a letter are significantly different from one another (ANOVA and Student's *t*-test for assessing effects of maternal status and cortisol treatment, respectively; see text for details); where no letters are present, no statistically significant differences were detected.

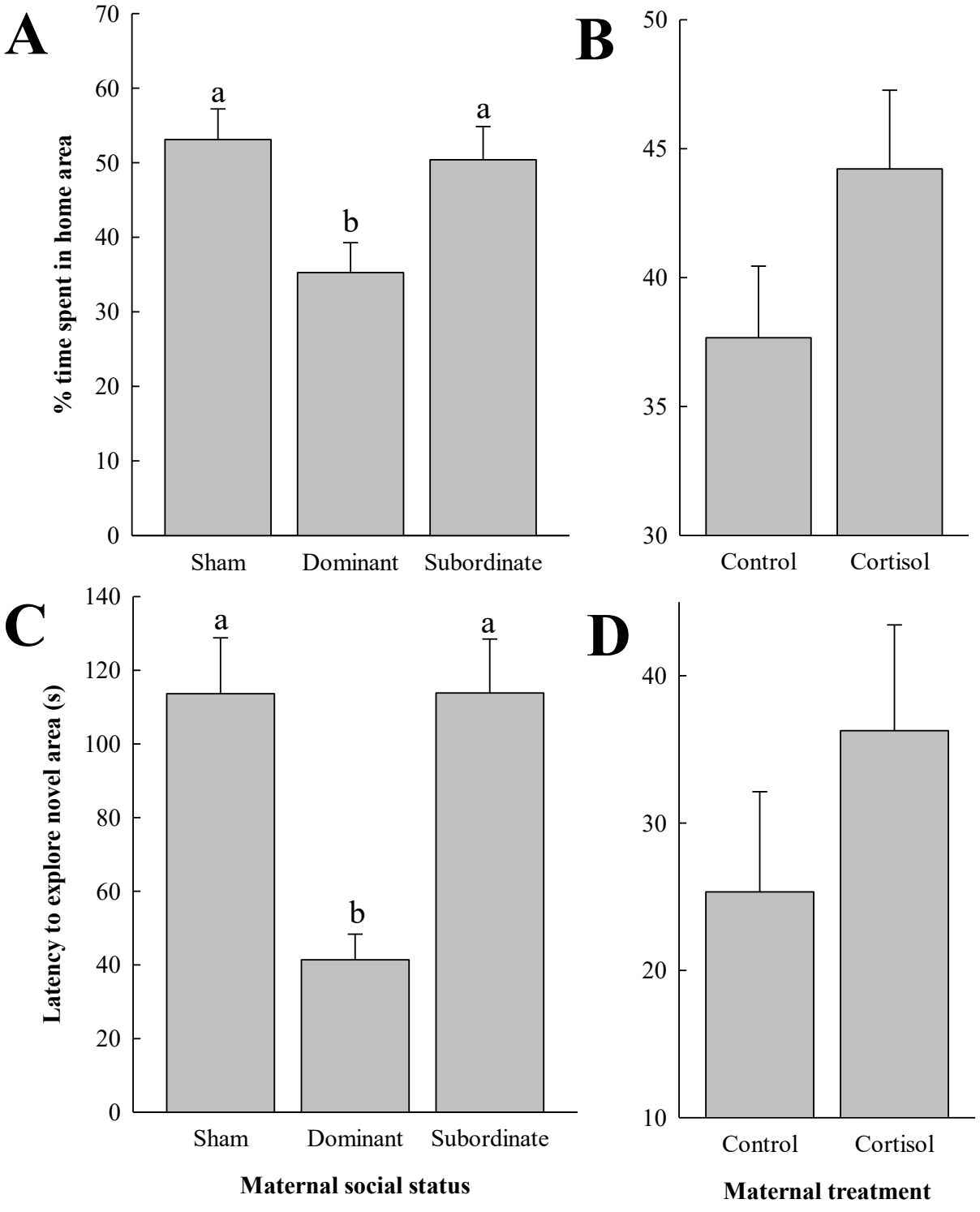
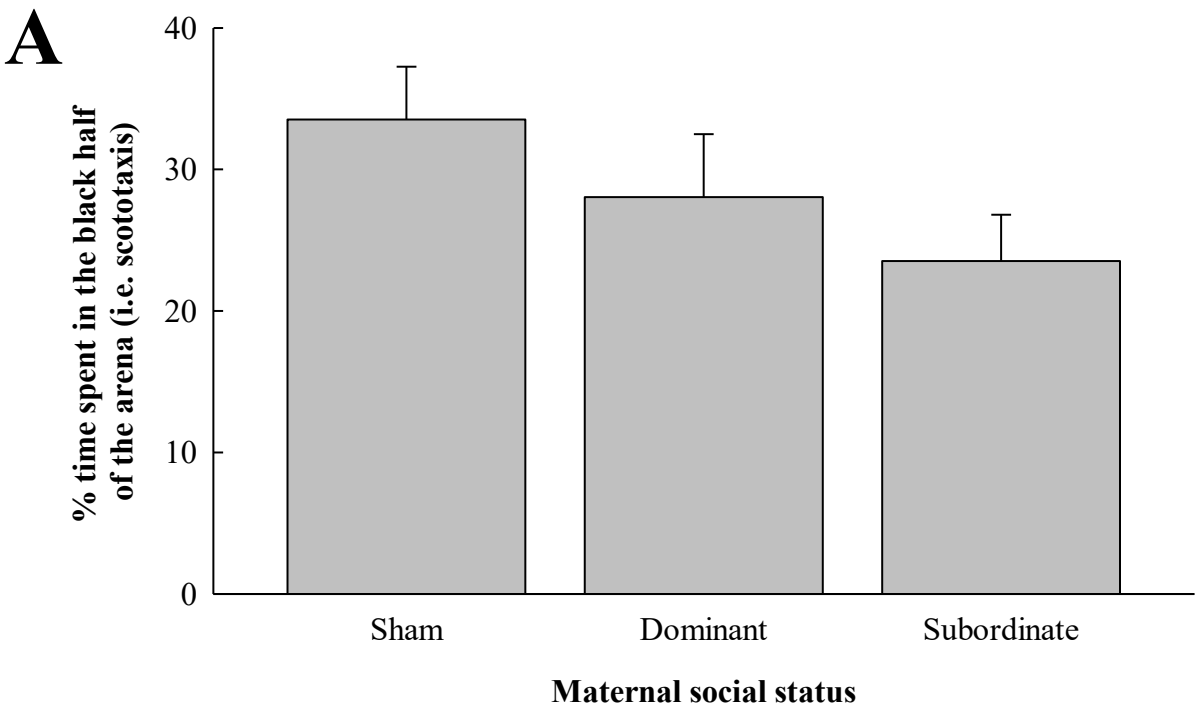

A
% time spent in home area
70
60
50
40
30
20
10
0
a
b
a
Sham
Dominant
Subordinate
B
50
45
40
35
30
Control
Cortisol
C
Latency to explore novel area (s)
140
120
100
80
60
40
20
0
a
b
a
Sham
Dominant
Subordinate
Maternal social status
D
40
30
20
10
Control
Cortisol
Maternal treatment

Figure 2.6

Figure 2.7 Effects of maternal social status (A) and maternal cortisol treatment (B) on the percentage of time 7 DPF zebrafish (*Danio rerio*) offspring spent in the black half of the test arena during the black-white preference test for scototaxic anxiety. Values are means + SEM. No significant differences in scototaxic anxiety were detected among offspring of sham (n = 61), dominant (n = 48), and subordinate (n = 57) females, or between offspring of control (n = 17) and cortisol-treated (n = 16) females (ANOVA and Student's *t*-test for assessing effects of maternal status and cortisol treatment, respectively; see text for details).


A
40
30
20
10
0
% time spent in the black half of the arena (i.e. scototaxis)
Sham
Dominant
Subordinate
Maternal social status

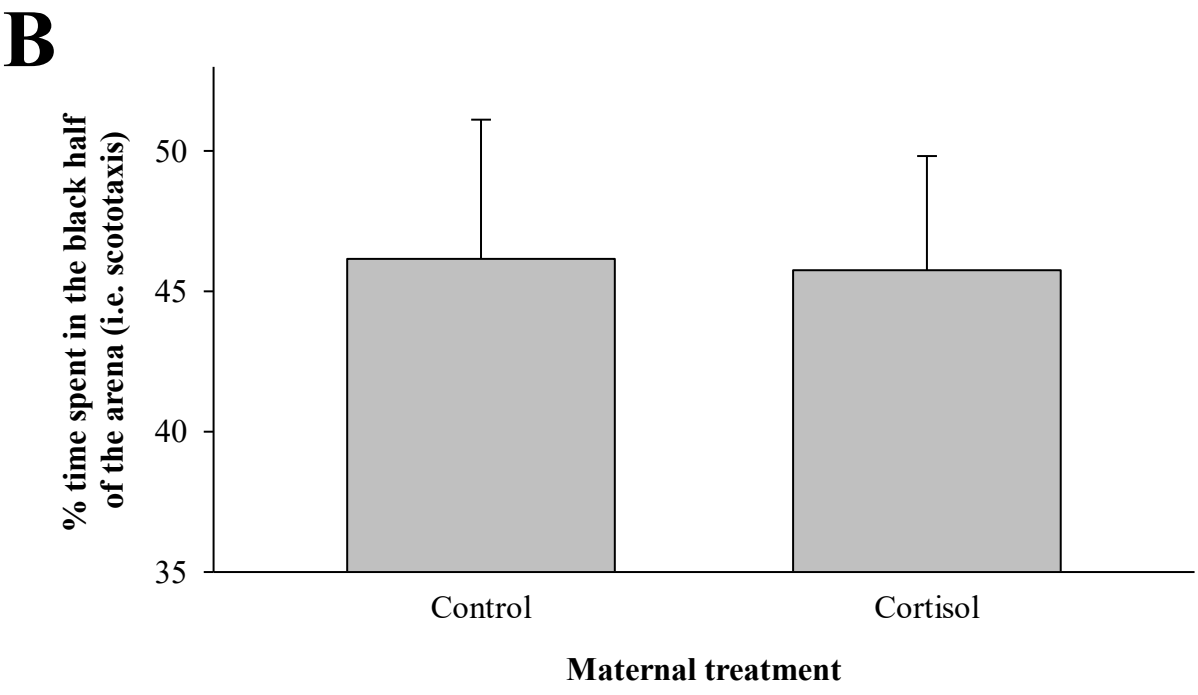

B
50
45
40
35
% time spent in the black half of the arena (i.e. scototaxis)
Control
Cortisol
Maternal treatment

Figure 2.7

2.5 DISCUSSION

The present study tested the hypothesis that maternal social status affects offspring survival, behaviour, and cortisol levels. The results of the present study provided some support for this hypothesis but unexpectedly, dominant maternal status, rather than the predicted subordinate status, affected offspring phenotype. For example, the novel tank diving test suggested that offspring of dominant mothers may exhibit a more proactive stress coping style, characterized by increased boldness and less anxiety-related geotaxic behaviour than offspring of subordinate and sham females. Although the effects of maternal social status were generally subtle, the most striking observation was that maternal exposure to dyadic social interactions in general, and not the resulting social status of the two females, had the most widespread effects on offspring. Specifically, compared to offspring of sham females, offspring of females that engaged in agonistic social interactions prior to spawn had greater survival at 1 DPF, exhibited reduced baseline cortisol concentrations as embryos and 30 DPF larvae, and displayed lower levels of activity. Moreover, maternal cortisol levels did not seem to explain these effects. These results may be interpreted to suggest that maternal social interactions prior to spawn prime offspring for an environment in which the offspring are likely to encounter high levels of exposure to stressors, therefore providing support for the hypothesis of transgenerational adaptive plasticity (Mousseau and Fox, 1998; Green, 2008) in a 'maternal match' situation (Breuner, 2008; Love et al., 2013). Here, the observed significant effects of maternal social interactions and dominant status on offspring will be discussed. In addition, offspring traits that were predicted to be affected by maternal status but were not, as well as possible sources of error, will be addressed.

2.5.a Effects of maternal social interaction on offspring phenotype

The predictions of the present study focused on likely differences in offspring between dominant and subordinate females, with the underlying assumption being that offspring of dominant and sham females would exhibit similar 'control' phenotypes. However, the results clearly suggest that both dominant and subordinate females experienced conditions, perhaps stress, that ultimately affected the survival, behaviour, and physiology of the next generation. Subordinate and dominant females, regardless of their resulting social status, engage in physical interactions that fish held alone in a tank (shams) do not experience. Behavioural observations recorded during interactions (data not shown; scoring exemplified in Appendix B) reveal that most attacks or chases by the dominant female were accompanied by retreat of the subordinate female. Both aggression and submission therefore require short bursts of energy and increased vigilance. These physical interactions may be accompanied by elevated stress levels. For example, in rainbow trout, another teleost species that readily forms social hierarchies following dyadic interactions, acute rises in plasma cortisol and catecholamine hormones have been documented during hierarchy formation (Øverli et al., 1999; Thomas and Gilmour, 2006). Whether the effects of maternal social interaction on offspring survival, cortisol, and activity levels observed in the present study stem from the single period of acute stress during hierarchy formation or exposure to repeated acute stressors involved in hierarchy formation and maintenance requires further investigation.

The existing literature on the effects of maternal stress and/or cortisol treatment in zebrafish focuses on offspring younger than 6 DPF (6 DPF, Jeffrey and Gilmour, 2016; 3 DPF, Nesan and Vijayan, 2012, 2016; Wilson et al., 2013), so the observation in the present study of

maternal experience effects to at least 30 DPF highlights the need for future studies to assess multiple life-stages. Similarly, few studies appear to have measured cortisol concentrations of zebrafish larvae and/or juveniles older than 10 DPF but prior to sexual maturity (70 DPF; Westerfield, 2007). Indeed, the present study may be the first to report cortisol levels of zebrafish larvae at 15 and 30 DPF. However, it should be noted that although the embryonic cortisol values reported in the present study (for both experiments) were similar to published values (0.67 ± 0.16 pg per embryo compared to ~0.75 pg per embryo in Alderman and Bernier, 2009, and ~1 pg embryo^{-1} in Jeffrey and Gilmour, 2016), the baseline cortisol concentrations of 6 DPF larvae reported in the present study were approximately ten-fold lower than those reported previously (0.45 ± 0.15 pg per larva compared to ~3.5 pg per larva in Jeffrey and Gilmour, 2016, and ~4 pg per larva in De Marco et al., 2013). Despite numerous troubleshooting attempts, larval cortisol levels in the present study remained unusually low compared to published values, albeit consistently low across all treatment groups. Regardless of the absolute cortisol values, stress-induced whole-body cortisol concentrations observed in the present study were significantly higher than baseline cortisol concentrations in all cases, thus validating the use of the air exposure stressor. Maternal social experience affected baseline rather than stress-induced cortisol levels, in contrast to the finding of Jeffrey and Gilmour (2016) that offspring of subordinate females exhibited reduced stress-induced cortisol concentrations, but in partial agreement with their finding that offspring of dominant females exhibited decreased baseline cortisol at 2 DPF. However, it should be noted that the acute stressor (swirling instead of air exposure) and method of fertilization (*in vitro* instead of natural breeding) were different between the two studies, potentially accounting for differences in results.

In addition to lowered baseline whole-body cortisol concentrations at 0 and 30 DPF, offspring of females that engaged in social interactions exhibited significantly better (15% higher) survival at 1 DPF compared to offspring of sham-treated females. This finding is in accordance with previous studies in teleosts that have reported reduced survival of embryos directly exposed to exogenous cortisol (e.g. Gagliano and McCormick, 2009). Whether decreased cortisol levels play a causal role in allowing improved survival in embryos requires further investigation. It should be noted, however, that Nesan & Vijayan (2016) reported an increase in the frequency and severity of morphological defects in embryos that were microinjected with a cortisol antibody at the single-cell stage to sequester maternally deposited embryonic cortisol, suggesting that substantially reduced embryo cortisol may be disadvantageous. Together with the finding in the present study of improved survival in embryos with small but significant reductions in cortisol content, these studies suggest that tight regulation of maternal cortisol deposition is essential for proper embryogenesis and survival.

The effects of maternal social experience on offspring cortisol and survival observed in the present study cannot be attributed to maternal cortisol, because treatment of adult females with exogenous cortisol prior to spawn did not affect offspring survival or cortisol levels. Similarly, Jeffrey and Gilmour (2016) detected effects of maternal social status on offspring HPI axis activity in the absence of differences in maternal cortisol concentrations. Together, these results highlight the need for future studies to explore mediators of maternal effects beyond maternal cortisol, such as altered yolk provisions (altered yolk composition or overall volume of yolk provisioned) or epigenetic effects.

Maternal dyadic social interactions also resulted in offspring that displayed a lower activity level in an open-field test at 6 DPF. Specifically, these larvae spent a smaller percentage of time actively swimming owing to longer periods of immobility, resulting in a shorter overall distance travelled. An abrupt increase in light levels triggers a marked reduction in the activity of zebrafish larvae, with activity gradually increasing over ~20 min when the high light level is sustained (e.g. MacPhail et al., 2009). The 5 min recording period used in the present study emphasized the freezing response to the onset of bright light, and therefore differences in activity during this period may be indicative of differences in activity-based anxiety and/or the strength of the fear response. On average, larvae in the present study travelled 15.5 ± 2.4 cm in 5 min, which is consistent with a previous study in which larvae travelled ~10-22 cm (in 5 min) when exposed suddenly to bright conditions (Best, 2015). Offspring of sham females engaged in active swimming about three times more than offspring of females that engaged in social interactions, and also tended to travel greater distances in the present study. These observations suggest that offspring of mothers that interacted socially were more anxious and/or fearful (in response to bright lighting conditions) than offspring of sham-treated females. Notably, embryos and 30 DPF larvae of mothers that interacted socially also exhibited lower cortisol levels than the corresponding offspring of sham females, although no difference was present at 6 DPF when the open-field test was carried out. Interestingly, Best (2015) reported a positive correlation between embryo cortisol concentrations and larval activity; zebrafish embryos treated with exogenous cortisol later exhibited increased larval activity levels in response to bright light in an open-field test. The work of Ziv et al. (2013) on a zebrafish mutant lacking transcriptional activity of GR provides further support for a role of cortisol together with GR signalling in mediating anxiety-related behaviour in fish; the mutants exhibited increased freezing behaviour in adult fish. The

mechanisms through which cortisol affects behaviour remain to be elucidated (reviewed by Egan et al., 2009).

2.5.b Impact of maternal dominant status on offspring stress-coping style

In contrast to most of the significant effects on offspring traits, which were associated with maternal social interactions, offspring behaviour in the novel tank diving test reflected maternal social status. That is, offspring of dominant females exhibited greater boldness and less geotaxic anxiety in the novel tank diving test, characterized by a shorter latency to exit the home area to explore a novel environment (65% shorter latency) and a smaller percentage of time spent in the home area (20% less), than offspring of sham or subordinate females. This difference in offspring behaviour occurred in the absence of differences in cortisol concentrations between offspring of dominant and subordinate females, suggesting that factors other than (offspring) cortisol concentrations contribute to behavioural phenotypes. However, offspring of control, untreated females tended to exhibit greater boldness in the novel tank diving test than offspring of cortisol-treated females, suggesting a dampening effect of elevated maternal cortisol on offspring boldness. It is likely that behavioural phenotypes are determined by multiple, interacting factors. For example, behavioural phenotypes indicative of anxiety in studies of adult zebrafish in the novel tank diving test (e.g. longer freezing bouts and more bottom-dwelling behaviour) can be modulated by the presence of various compounds leading to increased or decreased anxiety, such as caffeine or skin alarm pheromones (anxiogenic) and fluoxetine or ethanol (anxiolytic), respectively (reviewed by Egan et al., 2009). Although studies have measured anxiety-related behaviour in zebrafish larvae in the open-field (Ahmad and

Richardson, 2013) and black-white preference (Maximino et al., 2010; Watkins et al., 2004) tests, the present study appears to be the first to report the anxiety-related behaviour of larval zebrafish within the novel tank diving test.

The effect of maternal dominant status in lowering offspring geotaxic anxiety during the novel tank diving test may be linked to the observation that larvae of dominant females exhibited greater boldness to explore to a novel area sooner. Because zebrafish larvae at 6 DPF, regardless of maternal status, exhibited low levels of active swimming (~1% of the observation period; ~3 s of active swimming in total), the offspring of dominant females may have spent less time in the home area because they left the home area earlier but then did not engage in enough active swimming to return. Further investigation of this finding is required. Possible next steps could include assessment of the distribution of active swimming over time and space.

Interpretation of the effect of maternal status on geotaxic anxiety is further complicated by the lack of consistent effects on offspring anxiety across the open-field, light-dark preference, and novel tank diving tests. The differing observations from the open-field test (offspring of females that had socially interacted had a stronger fear response) versus the novel tank diving test (offspring of dominant females were less anxious and more bold) emphasize the caution that must be used in drawing conclusions from these tests of behaviour. Furthermore, results for the light-dark preference test of scototaxis may have been affected by lighting conditions, and thus should, at best, be considered with caution if not disregarded completely (see Ch. 4). Nevertheless, the observations that offspring of dominant females exhibited greater boldness and less anxiety in the novel tank diving test suggest that maternal social dominance may affect offspring stress-coping style. That is, dominant females may produce offspring that exhibit

behavioural phenotypes characteristic of a more proactive stress-coping style. However, a proactive stress-coping style is usually associated with a reduced cortisol response to stress (De Boer et al., 1990; Korte et al., 1992; Fokkema et al., 1995), and the present study did not reveal an effect of maternal social experience on offspring stress-induced cortisol levels. Regardless, whether offspring with a more proactive stress-coping style experience increased fitness is likely environmentally- and socially-dependent and is a question that requires investigation. Indeed, further work is also required to determine whether maternal social dominance or social interactions in general, regardless of resulting social status, prime offspring to better perform in an environment where they experience stressor exposure comparable to that of the maternal environment, i.e. a matched maternal-offspring environment (Breuner, 2008; Love et al., 2005, 2013). If there are offspring fitness consequences associated with maternal social interactions and/or resulting status, the potential exists for maternal social experience to affect future population dynamics via transgenerational adaptive plasticity (Mousseau and Fox, 1998; Green, 2008).

CHAPTER 3

EFFECTS OF MATERNAL CORTISOL TREATMENT ON OFFSPRING RESPONSES TO STRESS AND ANXIETY-RELATED BEHAVIOUR IN WILD LARGEMOUTH BASS (*Micropterus salmoides*)

Note on Ch. 3

Ch. 3 is in preparation to be submitted to the peer-reviewed journal *Hormones & Behavior* with the following citation:

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PREFACE

Contributions of authors

JR was responsible for data collection and analysis, as well as manuscript composition with the help of KMG. All authors contributed to concept development and experimental design. In addition, MAN and RJL assisted in the collection of bass from surrounding lakes, and with treatment of bass with cortisol. SJC provided field equipment, including vehicle transportation. RJL, SJC, and KMG assisted with the statistical analysis of data.

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John English and members of the Kaskaskia field station (Illinois Natural History Survey) provided assistance with adult bass collection in fall 2013 and spring 2014, and the former also helped with cortisol treatment and stocking of adult largemouth bass into experimental ponds in April 2014. Brian Van Ee, Mitch Stanton, and Benjamin Smith collected bass young-of-the-year by seining in June 2014. Brett Culbert, Olivia Gallarino, Katie Wiwchar, Rowan Harris, Chantal Williams, and Daniel Kostyniuk aided in scoring bass behaviour from video recordings, and Benoit Tremblay helped to write the MATLAB script used to quantify thigmotaxis in the open-field test. The present research was supported by NSERC Discovery Grants to KMG and SJC. SJC holds an NSERC Canada Research Chair. JR was supported by an NSERC Alexander Graham Bell Canada Graduate Scholarship-Master's (2014), and an Ontario Graduate Scholarship (2015).

Ethics statement

Work with live largemouth bass was conducted at the Sam Parr Biological Station, Illinois Natural History Survey (Kinmundy, Illinois, USA) from March to June 2014. Field methods complied with the guidelines of the Canadian Council on Animal Care for the use of animals in research and teaching. Experimental protocols were approved by the University of Ottawa (protocol BL-2118) and Carleton University (protocol B10-09), respectively.

3.1 ABSTRACT

Cortisol, the main GC stress hormone in teleost fish, is of interest as a mediator of maternal stress on offspring characteristics because cortisol plays an organizational role during early development. The present study tested the hypothesis that maternal exposure to exogenous cortisol prior to spawn affects offspring phenotype using wild largemouth bass (*Micropterus salmoides*). Baseline and stress-induced cortisol concentrations, length and mass, and behaviour (anxiety, exploration, boldness, and aggression) were assessed at different offspring life-stages and compared between offspring of control and cortisol-treated females. Exogenous cortisol administration did not affect spawning success or timing, nor were whole-body cortisol concentrations different between embryos from cortisol-treated and control females. However, maternal cortisol treatment had significant effects on offspring stress responsiveness, mass, and behaviour. Compared to offspring of control females, offspring of cortisol-treated females exhibited larger mass right after hatch (2-4 DPF), and young-of-the-year (35-50 DPF) mounted an attenuated cortisol response to an acute stressor and exhibited less boldness and thigmotaxic anxiety, but greater fear-related behaviour. The observation that offspring phenotype was affected by elevated maternal cortisol levels despite the absence of a significant increase in embryo cortisol concentrations suggests that a mechanism other than the direct deposition of cortisol into eggs mediates effects on offspring. The results of the present study provide support for the hypothesis of transgenerational adaptive plasticity, because the observed effects of elevated maternal cortisol prior to spawn could conceivably confer higher fitness to offspring in a stressful environment, if the environment of the progeny matched that of the mother.

3.2 INTRODUCTION

Fish may be exposed to a gauntlet of natural and anthropogenic stressors, including but not limited to angling, over-fishing, physical habitat loss, changes in water flow, exposure to chemical and noise pollution, invasive species, disease, and climate change, many of which are increasing in frequency and intensity (reviewed by Dudgeon et al., 2006). These stressors have been linked to population declines in fish (Kappal, 2005), and contribute to the current high rate of biodiversity loss and extinction in freshwater fish (Ricciardi and Rasmussen, 2001). Although a large body of literature explores the effects of exposure to such stressors on adult and even larval fitness-related traits in fish (e.g. reviews by Bartholomew and Bohnsack, 2005; Lafferty and Kuris, 1999; Slabbekoom et al., 2010), less effort has been invested in studying intergenerational effects of stressor exposure. This gap in knowledge should be addressed because the propagation of a population relies on the survival and successful reproduction of offspring.

From an applied perspective, it is important to conduct research asking such conservation-related questions in the field, because laboratory or farmed fish are exposed to different types of stressors than wild animals (e.g. frequent handling vs predation, respectively) and therefore, it is difficult to apply predictions from laboratory studies to animals in their natural environment (Calisali and Bentley, 2009). For example, largemouth bass (*Micropterus salmoides*) is the most popular freshwater gamefish species, as evidenced by the multibillion dollar freshwater bass fishing industry worldwide (Williamson, 2016), so it is a species that is exposed to intense angling stress in the wild. However, it is not commonly farmed in Canada (Brown et al., 2009). Thus, field studies are needed to support conservation and fisheries

management goals for largemouth bass. Besides being economically and culturally important, largemouth bass are also ecologically important because they have successfully invaded many bodies of water and have been associated with the decline of native fish species upon which they prey (Kerr and Grant, 1999), and because they serve as a form of biocontrol of invasive prey items (e.g. round goby, *Neogobius melanostomus* in the Great Lakes; Corkum et al., 2004). Although previous studies have explored the effects of stressors on adult largemouth bass (e.g. Cooke et al., 2000; reviewed by Siepker et al., 2007), the impact of these stressors on the next generation has received little attention to date.

Intergenerational effects of stress prior to spawn largely have been investigated with respect to the female parent because the female provisions the developing embryo with an egg yolk into which maternal mRNA, proteins, lipids, and hormones are deposited during vitellogenesis (Mousseau and Fox, 1998). Pre-spawn maternal stress has been reported to affect offspring survival, morphology, physiology, and behaviour. Broadly, the reported effects of maternal stress in teleosts include smaller embryos and larval length, and reduced responsiveness to stress, boldness, and ability to learn (McCormick, 1998; McGhee et al., 2012; Jeffrey and Gilmour, 2016; Roche et al., 2012). However, effects on other variables such as survival (Patterson et al., 2004; but see Sopinka et al., 2014), clutch size (Mileva et al., 2011; but see McCormick, 2006), embryo cortisol levels (Stratholt et al., 1997; but see Sopinka et al., 2014), and larval anti-predator behaviour (Giesing et al., 2011; but see McGhee et al., 2012) were variable in occurrence because effects of maternal stress appear to depend on the type and intensity of the stressor, the duration of exposure, the environment in which the female was exposed to the stressor, the phase of oogenesis during which the female was exposed to the stressor, and the offspring life-stage assessed. Despite these sources of variation, the literature

reports many similar effects on offspring of maternal stress prior to fertilization, independent of the type of stressor. For example, rainbow trout (*Oncorhynchus mykiss*) females subjected to repeated air exposure (Campbell et al., 1992), confinement (Campbell et al., 1994), or chasing, crowding, and noise (Contreras-Sanchez et al., 1998) all reared significantly smaller embryos. These observations raise questions about what aspect of the maternal stress response mediates such intergenerational effects.

When a fish is exposed to a stressor, the HPI axis is activated, culminating in the increased production of cortisol (Wendelaar Bonga, 1997). This GC stress hormone is of interest as a mediator of maternal stress effects on offspring because cortisol is among the maternal provisions deposited into developing eggs (Brooks et al., 1997), and it plays an organizational role during early development in teleost fish, affecting embryonic development of the eye and HPI axis, the formation of skeletal and cardiac muscle, and neurogenesis (Nesan and Vijayan, 2012, 2016). Furthermore, in at least some fish species, egg cortisol concentrations are positively correlated with circulating maternal plasma cortisol concentrations (Stratholt et al., 1997; Eriksen et al., 2006 but see Faught et al., 2016).

To investigate the role of cortisol in mediating effects of maternal stress, previous studies have treated fertilized eggs with exogenous cortisol (Nesan and Vijayan, 2012; Sampath-Kumar et al., 1993). Effects on offspring have included malformations (Nesan and Vijayan, 2016), a reduced or absent cortisol response to an acute stressor (Auperin and Geslin, 2008; Nesan and Vijayan, 2016), and less aggression (Burton et al., 2011 but see Sloman, 2010) and boldness (Wilson et al., 2013). Although treating eggs with exogenous GCs is a logistically simple approach, it does not take into account other possible maternal effects following stressor

exposure, such as ovarian follicle buffering against egg hypercortisolism (Li et al., 2012; Faught et al. 2016), altered egg provisions (Henriksen et al., 2011), or epigenetic effects (Szyf et al., 2005; Mommer and Bell, 2014). As such, experimental elevation of maternal cortisol rather than direct exposure of eggs may be a more appropriate proxy for maternal stress. Few studies have adopted this approach, let alone in a field setting (Eriksen et al., 2006, 2011; McConnachie et al., 2012; McCormick, 1998).

O'Connor et al. (2013) exposed wild, adult female largemouth bass to exogenous cortisol via a cortisol-infused cocoa butter implant prior to spawn, and reported reduced energetic stores and elevated circulating plasma cortisol levels relative to untreated control females. Importantly, O'Connor et al. (2013) also observed ovarian cortisol concentrations in treated females that were higher than those in untreated control fish even 9-13 days post-injection (DPI). Thus, the purpose of the present study was to build upon the work of O'Connor et al. (2013) and test the hypothesis that experimentally elevated maternal cortisol affects offspring phenotype in wild largemouth bass. The effects of maternal cortisol treatment were assessed by measuring the offspring growth, the cortisol response of offspring to an acute stressor, and anxiety-related behaviour at different developmental stages. Based on previous research, we predicted that offspring of cortisol-treated, wild, female largemouth bass would be smaller than those of control females, they would exhibit a reduced cortisol response to an acute air stressor, and they would exhibit greater anxiety, including more thigmotaxis (wall hugging) and scototaxis (choice of dark environments), greater freezing behaviour, reduced boldness, and reduced aggression.

3.3 MATERIALS AND METHODS

3.3.a Study site and animals

The present study was conducted at the Sam Parr Biological Station (38°43'N, 88°45'W; Illinois Natural History Survey) using wild adult largemouth bass collected from nearby lakes. Adult largemouth bass (>30 cm in length; n = 34 males and 25 females; mean mass ± SEM = 500 ± 43 g) were collected on April 1st and 2nd, 2014, from Forbes Lake (38°71'N, 88°75'W) and Lake Shelbyville (39°47'N, 88°71'W) in Illinois, USA by electrofishing, and then transported in 500 L insulated tanks to the Sam Parr Biological Station, where they were held in 400 L tanks supplied with aerated fresh water from the adjacent Forbes Lake for 2-3 days to recover. Tanks were covered with a net and housed under an open-sided shelter.

On April 4th, 2014, fish were individually netted from the holding tanks, weighed, and sexed as per Benz and Jacobs (1986). Male bass were distributed equally by number and weight into six experimental ponds (4-7 males per pond). Female bass were alternately assigned to either control or cortisol treatment groups (three ponds per maternal treatment group, 4-5 females per pond). The six 0.4 hectare research ponds were man-made and drainable, yet they were located outdoors and contained natural earthen sediment exposed to weather and predators, thus allowing the present experiment to be conducted in a semi-natural environment.

Females assigned to the cortisol treatment group were placed in a water-filled trough with their ventral side exposed and given a 5 ml kg^{-1} intraperitoneal injection of 10 mg ml^{-1} hydrocortisone 21-hemisuccinate (H4881 Sigma-Aldrich) emulsified in melted cocoa butter (NOW, Herb n' Spice Shop, Ottawa, ON). O'Connor et al. (2013) reported that the rise in

circulating plasma cortisol concentrations following cortisol treatment was comparable to the endogenous levels following ecologically-relevant stressors such as catch-and-release angling, confinement, and exhaustive exercise (O'Connor et al., 2009). Also, importantly for the present study, O'Connor et al. (2013) reported elevated ovarian cortisol concentrations 9-13 DPI, thus validating the protocol for the purpose of the present experiment.

Female bass assigned to the control treatment were left untreated. Previous studies in teleosts have reported that sham-treated fish (i.e. vehicle injection only) may exhibit circulating cortisol concentrations similar to those of controls as well as those of cortisol-treated fish (McCormick, 1998; DiBattista et al., 2005; O'Connor et al., 2009; 2010; 2011; Dey et al., 2010). For this reason, and for consistency with O'Connor et al. (2013), an untreated control group rather than a sham-treated group was included in the experimental design.

3.3.b Sampling and fish husbandry

Nest locations were determined by observing patrolling and guarding behaviour of male bass. Four life-stages of offspring were assessed (Brown et al., 2009): embryos (0-2 DPF; after spawn until hatch), egg-sac fry (ESF; 2-4 DPF; after hatch until swim-up), free-swimming fry (FSF; 5-~30 DPF; swim-up to paternal abandonment and/or brood dispersal), and young-of-the-year (YOY; paternal abandonment and/or dispersal to 1 year). From April 19th to May 2nd, embryos were collected using a turkey baster, while ESF and FSF were collected using a fine hand-held dip net. Later, YOY were collected (June 25-28th) via seine net. Offspring were euthanized for measurement of mass and length (n = 10 embryos and fry from each nest at 0 DPF and 2, 4, 6, 8, and 10 DPF, respectively, and 10 YOY at ~45 DPF from each pond), and whole-body cortisol concentrations (n > 24 embryos at 0 DPF and fry at 2-4 and 6-8 DPF from each

nest and 20 YOY at 35-50 DPF from each pond). A third subset of FSF (>15 fry per nest, tested at 8-12 DPF) and YOY (>60 per pond; tested at 35-50 DPF) was brought into the field station and held until fish were subjected to behavioural tests (one test per individual).

The FSF and YOY collected for behavioural tests were brought into the Sam Parr Biological Station laboratory and allowed to recover for at least 24 hours before being subjected to a behavioural test. The fry were held at ambient temperature for at least two days in mason jars (~20 fry per jar per nest) containing artificial pond water made by mixing tap water with a commercial product that removes iron from tap water and inhibits fungal and bacterial blooms (Jungle "Pond Water Clear", Walmart, Salem, Illinois). Water was changed daily (2/3 water change), and FSF were fed by adding 3 ml of zooplankton-rich pond water daily. Zooplankton were collected from the experimental ponds using a cone mesh zooplankton net every 2-3 days, and were held in mason jars under the same conditions as FSF. The YOY were held for approximately two weeks in 10 L tanks (10 fish per tank per pond, n > 60 per pond) containing aerated pond water changed every 4-5 days (2/3 water change). They were fed frozen bloodworms daily (Sally's Bloodworms, San Francisco Bay Brand, PetSmart, Champaign, Illinois), and were released into a nearby stream following completion of behavioural tests. For all holding tanks, excrement and any dead fry were removed daily.

3.3.c Assessment of offspring characteristics

Physiological and behavioural traits of the offspring were quantified and compared between control and cortisol-treated females.

3.3.c.i Growth

The length and mass of embryos, fry, and YOY were measured. Embryo and fry diameters and lengths, respectively, were quantified using ImageJ (Abramoff et al., 2004) from photographs taken under a dissecting microscope (10X) using a micrometer scale slide. The length of YOY was measured against a 30 cm ruler.

3.3.c.ii Cortisol responses to acute stress

Offspring collected from their resident pond were either immediately euthanized and flash frozen with liquid nitrogen (baseline cortisol levels) or were sampled following air exposure for 1 min and 5 min of recovery in a holding container filled with fresh pond water (stress-induced cortisol levels). The air exposure stressor and 5 min recovery period were chosen based on pilot studies using zebrafish larvae. Stress-induced cortisol concentrations were not measured for embryos because the fish species tested to date have not responded to external stressors prior to hatch (e.g. sea bass, *Dicentrarchus labrax*, Tsalafouta et al., 2014; zebrafish, Alderman & Bernier, 2009). Samples were transported to the University of Ottawa in a dry shipper and then stored at -80^{o}C for later analysis of cortisol concentrations.

Cortisol was extracted from embryos (yolk included) and fry using the protocol described by Jeffrey and Gilmour (2016). Whole-body cortisol concentrations were then quantified by enzyme-linked immunosorbent assay (EIA). In short, samples thawed on ice were homogenized on ice in 200 µL of 5X diluted extraction buffer from a commercial cortisol EIA kit (Cortisol EIA assay kit, #402710 Neogen Corporation—Life Sciences) using a battery operated pestle grinder (Kimble Chase Kontes). Homogenates were extracted thrice with 1 ml ether (anhydrous diethyl ether, #AC615080010 Fisher Scientific). After each addition of ether, samples were vortexed, incubated for 15 min (30 min the first time), centrifuged for 5 min at 3000 *g* at 4^{o}C,

flash frozen at -80°C, and decanted to transfer the cortisol-containing supernatant to a new 1.5 ml microtube. The ether extraction supernatants were combined and dried in the fume hood under forced air at room temperature. Lastly, the cortisol residue was reconstituted in 10 µL extraction buffer (Neogen) per embryo/fry in the original sample. To aid reconstitution, samples were heated for five min at 65°C and vortexed at least twice or until cortisol was completely dissolved. Cortisol was extracted from individual YOY using a protocol adapted for the larger mass of body tissue from the protocols of Sopinka et al. (2014) and Jeffrey and Gilmour (2016). The YOY were first powdered in liquid nitrogen with a mortar and pestle prior to homogenization with a handheld homogenizer (PowerGen 125, Fisher Scientific). Reagent volumes were scaled up from those used in the fry protocol based on the proportional increase in body tissue being processed. Extraction efficiencies determined by spiking homogenates with a known amount of radioisotope (^{3}H-hydrocortisone, 250 µCi, #NET396250UC Perkin Elmer) were 61.7% and 65.3% for embryos/fry and YOY, respectively. Extracts were stored at -80°C until quantification using a commercial EIA kit (Neogen). Samples were assayed in duplicate. Inter- and intra-assay variabilities were 5.8% and 2.5%, respectively.

3.3.c.iii Behaviour

Each individual was subjected to a single behaviour test to avoid habituation or stress associated with repeated handling. Between trials, the water in the test tank was changed. Fry were subjected to an open-field test or a black-white preference test. These tests also were conducted on YOY, as well as an emergence test, a predator recovery test, or a mirror test. For each test, the behaviour of five fry from each nest (five individual trials) and ten YOY per pond was recorded (Canon Vixia HF-R400 camcorder, 30 fps). Fish were recorded from above and all

test chambers had opaque walls to minimize disturbance from movement outside the tank. Fish were allowed to acclimate prior to recording for 2 min (Toms and Echevarria, 2014) in a starting chamber (PVC pipe, 3 cm diameter x 6 cm high for fry and 9 cm diameter x 10 cm high for YOY) for the open-field, black-white preference, and mirror tests; and for 5 min (Burns, 2008) on one side of the opaque divider for the emergence and predator exposure tests. The order in which individuals from each maternal treatment group as well as from each nest or pond for fry and YOY, respectively, were tested was randomized. Behavioural endpoints were extracted from videos of open-field tests using automatic tracking (see below), and behavioural endpoints were extracted from all other recordings by individuals who were blind to the treatment group to which the offspring belonged.

Open-field test (both FSF & YOY): This commonly used behaviour test assessing boldness and exploration (Burns, 2008; Toms and Echevarria, 2014) was conducted using an all-white test chamber (10 cm diameter x 3 cm high for fry and 14 cm diameter x 5 cm high for YOY), which maximized visual contrast, allowing automated tracking of video recordings using the tracking program idTracker (Pérez-Escudero et al., 2014). The steps involved in preparing the video recordings for automatic tracking and extraction of behavioural data from the resulting x and y coordinates (per frame, 30 fps) using MATLAB and Excel are described in Appendix F. After the two min acclimation period within the starting chamber (above), the chamber was removed and the test subject was recorded for five min. Total distance travelled, average velocity, percentage of time spent actively swimming (immobility defined as less than 1 mm of distance covered within one video frame), total number of freezes (a freeze defined as being immobile for at least 2 s/60 video frames), and percentage of time spent in the outer third of the test arena were quantified for each trial. The latter behaviour (% time in outer third of the test

arena) assessed an anxiety-related behaviour termed thigmotaxis (Levin and Cerutti, 2009; Schnörr et al., 2012), which is the tendency of an anxious animal to avoid open areas, preferentially situating itself in corners or along a wall (Nishio et al., 2001).

Black-white preference test (both FSF & YOY): This behaviour test assessed the anxiety-related behaviour of scototaxis (Maximino et al., 2010, 2011, 2012), which is defined as an anxious individual's preference for a dark environment (Maximino et al., 2010). In a round tank (10 cm diameter x 3 cm high for fry and 14 cm diameter x 5 cm high for YOY) in which one half of the tank was painted black and the other half, white, an individual test subject was acclimated for two min within the starting chamber. The acclimation chamber then was removed and the behaviour of the test subject was recorded for five min. The percentage of time spent in the black half of the tank was measured to assess scototaxis.

Emergence test (YOY): Exploratory and boldness traits (Réale et al., 2007) were assessed during an emergence test by quantifying the time taken by an individual fish to enter a novel environment as per Burns (2008) and Wilson et al. (2011, 2015). A single YOY was placed in a refuge separated from the main part of the experimental chamber (a 250 L glass aquarium) by an opaque, removable divider, and allowed to acclimate for five min. The divider was then lifted five cm and held in place by a remote pulley system, and the latency to emerge from the refuge was recorded to a maximum of 15 min, after which time the fish was recorded as having failed to emerge.

Predator recovery test (YOY): In a test chamber similar to that used for the emergence test, the test subject was again acclimated in the refuge for five min. The opaque removable divider was then lifted to completely reveal the other side of the tank, which contained a live

predator separated from the test subject by a static glass divider. The predator was an adult largemouth bass (a common nest predator; Post et al., 1998) angled from Forbes Lake, Illinois. Adult bass were held in a 60 L tank filled with aerated pond water, and were left to acclimate to the holding conditions for two days before behavioural trials commenced. The adult bass predators were not fed on the day of recording to maximize aggression towards the YOY on the other side of the glass divider. Typically, YOY freeze in response to the threat of a predator. The act of freezing in this predator visualization test assesses fear-related behaviour because the threat (i.e. predator) is imminent, as opposed to the freezing behaviour measured in the open-field test which is an anxiety-related behaviour because of the absence of any imminent threat (Steimer, 2002). After two min, the opaque divider was lowered and the latency of the YOY to begin moving was measured to a maximum of 10 min. A longer latency to move after freezing was indicative of greater fear.

Mirror test of aggression (YOY): In a 4.5 L tank with a mirror (8 cm wide and 12 cm high) attached to the inner side of one wall, the test subject was acclimated to the experimental chamber for two min with an opaque removable cover in front of the mirror. The cover was then removed, and behaviour was recorded for 25 min. Fish respond to their own reflection as if it were a conspecific, often exhibiting aggression (Gallup, 1968). The following behaviours indicative of aggression were measured: latency to approach the mirror, percentage of time spent charging or hitting the mirror, number of tail whips against the mirror, percentage of time spent tail whipping, latency to begin tail whipping, and total percentage of time spent exhibiting any form of aggression. This protocol was based on pilot trials (to determine length of recording) and adapted from other studies assessing aggression in different teleosts (e.g. daffodil cichlid,

Neolamprologus pulcher, Balzarini et al., 2014; mozambique tilapia, *Oreochromis mossambicus*, Ros et al., 2006).

3.3.d Statistical analyses:

Offspring traits were compared between control and cortisol-treated females. All data were expressed as mean values ± 1 SEM. All data analysis in the present study with bass was conducted using R (version R 3.1.1; R Core Team, 2013). The assumptions of normally distributed residuals and homoscedasticity were first tested for each measured behavioural and physiological trait in R using the Shapiro-Wilk Normality Test (Royston, 1995) and Bartlett's Test of Homogeneity of Variances (Bartlett, 1937). Where significant deviation from normal distribution of residuals was detected, the data were transformed using the ladder of powers (Velleman and Hoaglin, 1981) according to Kirchner (2001), in which case the minimum appropriate transformation required to achieve normality of the residuals was chosen. If no transformation could allow the data to meet all assumptions, then a non-parametric Mann-Whitney U rank sum test was used as an alternative. For the remaining normally distributed traits, the fixed effect of maternal cortisol treatment was assessed using a Student's *t*-test, except for cortisol concentrations, where the effects of both maternal treatment and air stressor exposure were evaluated in a two-way ANOVA. Where additional variables had to be taken into account as potential error the AIC of at least two models were compared: a general linear model testing only the fixed effect of maternal treatment on the response variable and at least one linear mixed effects model (nlme R package, Pinheiro et al., 2015) that incorporated any possible categorical variable as 'random effect(s)' such as nest/maternal ID or pond. Continuous variables that were potential sources of error were also taken into account by adding them as additional independent variables to the base model. If there was more than one applicable random effect, then multiple

mixed effects models were created in stepwise order. That is, variations of the base model plus each individual random variable separately were created, and then (an) additional model(s) combining the random effects were also included in the AIC model comparison. Candidate models were objectively compared using the Restricted Maximum Likelihood estimation to find the optimal random structure. The model with the lowest AIC value was deemed the best fit and was used to conduct a parametric test (Student's *t*-test comparing maternal treatments or ANOVA if continuous variables were included in the 'best' model). A significance value of $\alpha = 0.05$ was used for all statistical tests.

3.4 RESULTS

Control and cortisol-treated females did not differ significantly in spawning date (Fig. 3.1.A; one-tailed Student's *t*-test; P = 0.133), nor did their embryos differ significantly in cortisol concentrations (Fig. 3.1.B; one-tailed Student's *t*-test on reciprocal transformed data; P = 0.197). All spawning events occurred 13-28 days post-treatment. Neither pond nor the number of females, males, or total fish in the pond affected the spawning date of the adult largemouth bass (data not shown). There was also no effect of female cortisol treatment on the spawning success within ponds; 74.6 ± 13.0% and 79.4 ± 10.4% of males in the ponds containing control and cortisol-treated females, respectively, had eggs in their nests (Student's *t*-test; n = 3 ponds per maternal treatment group; P = 0.789).

Maternal cortisol treatment significantly influenced ESF mass right after hatch but was otherwise without effect on offspring length or mass (Fig. 3.2; Student's *t*-test at each life-stage;

P > 0.05 at each life-stage; see Appendix G for full statistical results). That is, ESF of cortisol-treated females had significantly higher mass compared to those of control females (Fig. 3.2.C, ESF 1 life-stage; Student's *t*-test; P = 0.011).

No significant effect of maternal cortisol treatment or air exposure was detected on the whole-body cortisol concentrations of ESF (Fig. 3.3.A; two-way ANOVA; P = 0.825 for MT, P = 0.492 for air, P = 0.497 for MT x air) and FSF (Fig. 3.3.B; two-way ANOVA; P = 0.655 for MT, P = 0.126 for air, P = 0.654 for MT x air). However, YOY exhibited significantly higher cortisol concentrations when exposed to the stressor, and the stress-induced cortisol concentrations of YOY of cortisol-treated females were significantly attenuated compared to YOY of control females (Fig. 3.3.C; two-way ANOVA, square root transformed data, pond included as a random effect; P = 0.504 for MT, P < 0.0001 for air, P = 0.045 for MT x air).

The behavioural traits of boldness, latency to move following an acute stressor, and thigmotaxic anxiety (i.e. tendency to hug the wall) in YOY were significantly affected by maternal treatment. Specifically, during the emergence test, YOY of cortisol-treated mothers took significantly longer to emerge from the refuge into the novel environment (Fig. 3.4.A; Student's *t*-test; P = 0.002) and also failed to emerge during the 15 min trial significantly more often (Fig. 3.4.B; Student's *t*-test; P = 0.019) than offspring of control females. During the predator exposure recovery test, YOY of cortisol-treated females exhibited a significantly longer latency to first move (Fig. 3.5.A; Mann-Whitney U rank sum test; P = 0.027) and similarly, were significantly more likely to remain immobile for the entire 10 min trial (Fig. 3.5.B; Student's *t*-test; P = 0.011) than offspring of control females. Whereas 21.4% of the YOY of cortisol-treated females maintained immobility for entire the observation period, all offspring of control females

recovered behaviourally from the acute stress of predator exposure during the 10 min behaviour trial. Finally, YOY of cortisol-treated females spent significantly less time in the outer ring of the open-field testing arena compared to YOY of control females, and thus exhibited significantly less thigmotaxic anxiety (Fig. 3.6.B; Student's *t*-test; P = 0.016); a similar trend was apparent in FSF (Fig. 3.6.A; Student's *t*-test; P = 0.084). Other than thigmotaxis, YOY activity (distance travelled, average velocity, number of freezing bouts, and percentage of time spent active) during the open-field test did not differ between maternal treatments for either life-stage tested (Table 3.1 for FSF and Table 3.2 for YOY). Similarly, the black-white preference test and the mirror test did not detect any significant effect of maternal cortisol treatment on offspring scototaxic anxiety (i.e. preference for black half of behavioural chamber; Table 3.1 for FSF and Table 3.2 for YOY) or aggression (Table 3.2), respectively.

Figure 3.1 Effects of maternal cortisol treatment on timing of spawning (A) and whole-body embryo cortisol concentrations (B; picograms, pg) of control versus cortisol-treated adult female largemouth bass (*Micropterus salmoides*). Values are means + SEM. Spawning timing was defined as the mean number of days after treatment with exogenous cortisol (i.e. DPI) before nests were observed (n = 11 control and 11 cortisol-treated females). Embryo cortisol content was determined for n = 4 control and 8 cortisol-treated females' clutches. No significant differences were detected (see text for details).

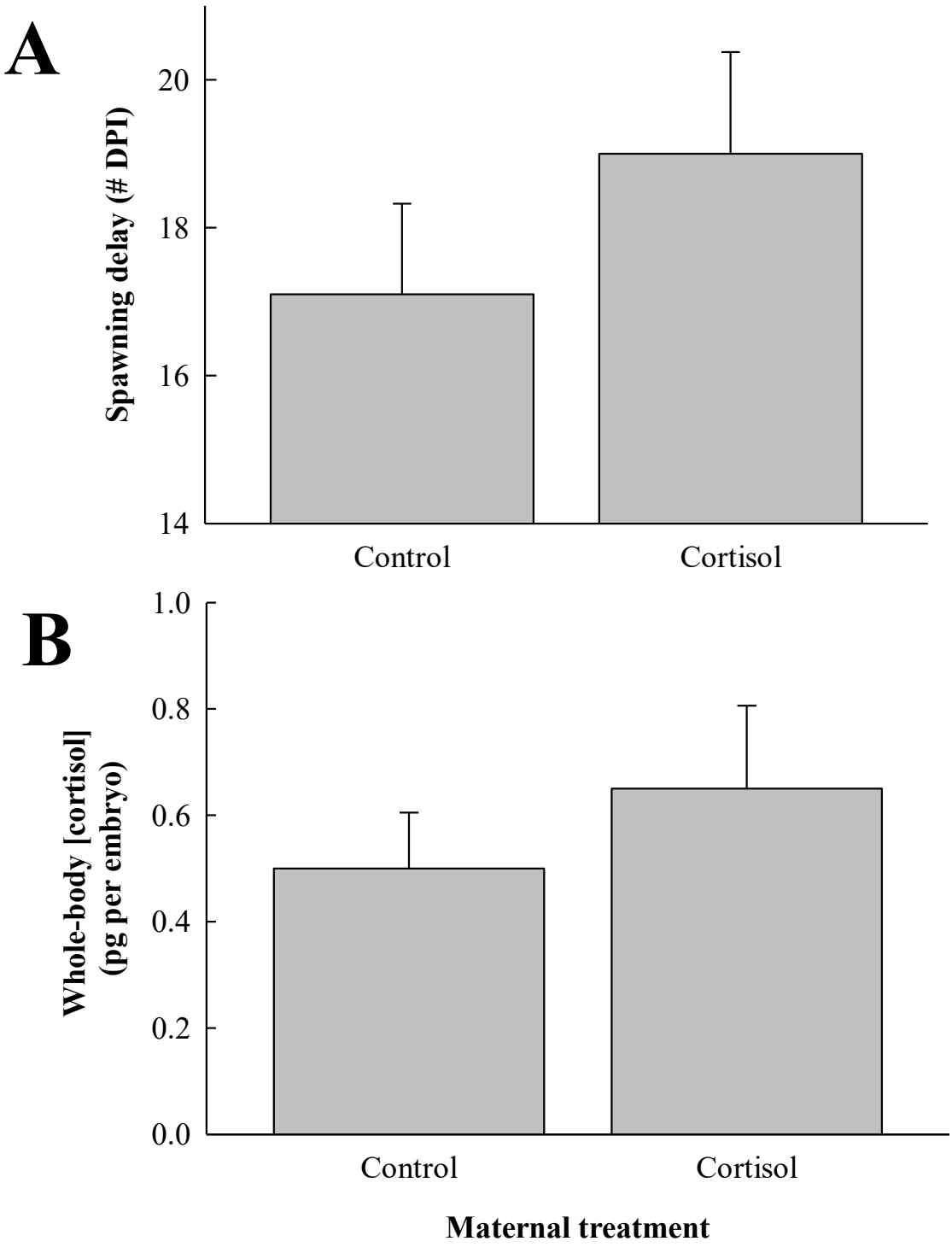
A
Spawning delay (# DPI)
20
18
16
14
Control
Cortisol
B
Whole-body [cortisol]
(pg per embryo)
1.0
0.8
0.6
0.4
0.2
0.0
Control
Cortisol
Maternal treatment

Figure 3.1

Figure 3.2 Individual offspring length (A and B) and mass (C and D) for offspring of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*) at different early life stages: embryo (0 DPF; n = 6 and 7 for offspring of control and cortisol-treated females, respectively), early egg-sac fry (ESF-1; 2 DPF; n = 5 and 7), late egg-sac fry (ESF-2; 4 DPF; n = 4 and 7), early free-swimming fry (FSF-1; 6 DPF; n = 9 and 9), and late free-swimming fry (FSF-2; 8-9 DPF; n = 6 and 9) in panels A (length) and C (mass); as well as YOY (35-50 DPF; n = 30 and 30) in panels B (length) and D (mass). Values are means + SEM. An asterisk represents a significant difference between maternal treatments within a life-stage (Student's *t*-tests; see text for details).

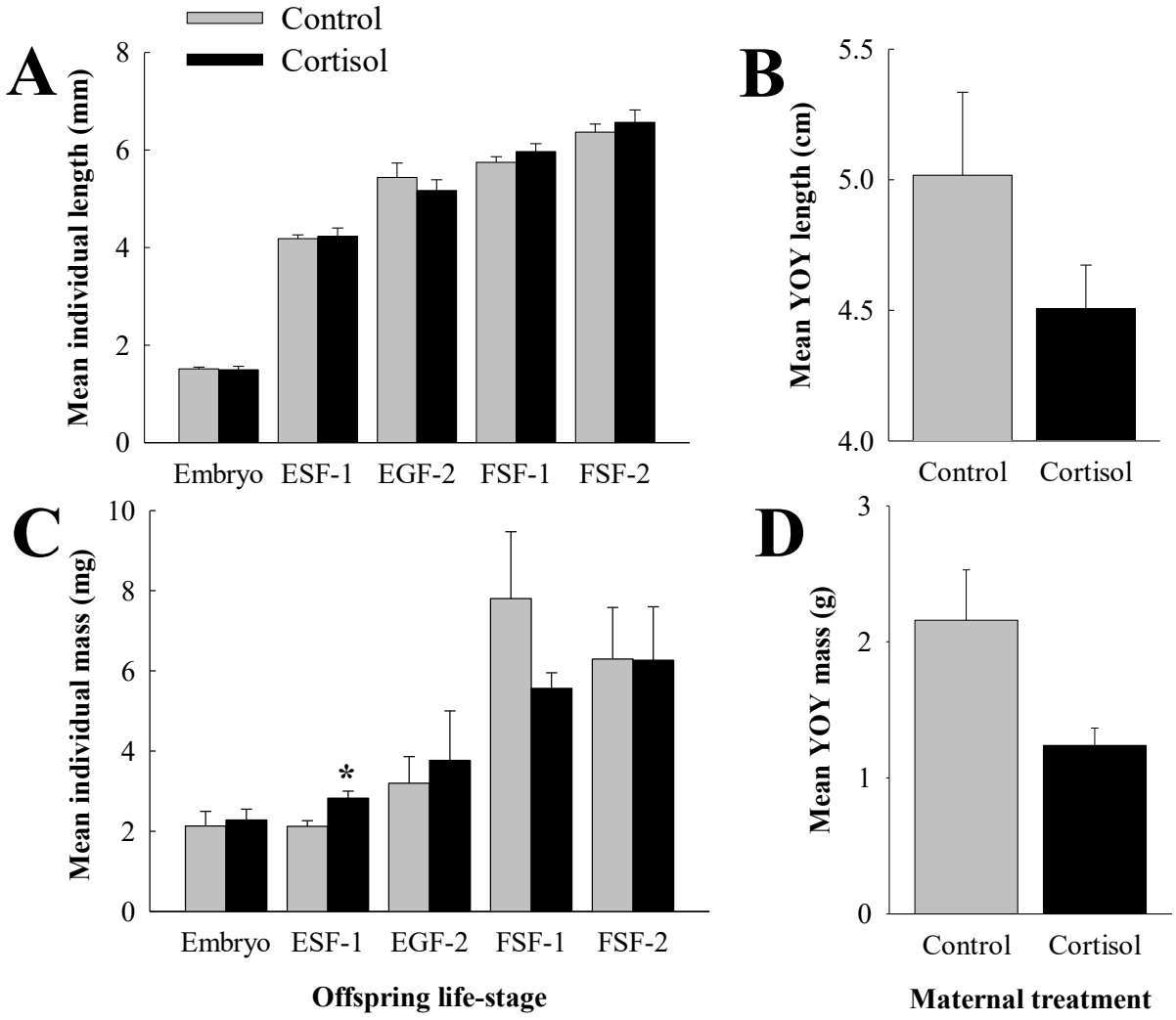
A
Control
Cortisol
Mean individual length (mm)
0
2
4
6
8
Embryo
ESF-1
EGF-2
FSF-1
FSF-2
B
Mean YOY length (cm)
4.0
4.5
5.0
5.5
Control
Cortisol
C
Mean individual mass (mg)
0
2
4
6
8
10
*
Embryo
ESF-1
EGF-2
FSF-1
FSF-2
Offspring life-stage
D
Mean YOY mass (g)
0
1
2
3
Control
Cortisol
Maternal treatment

Figure 3.2

Figure 3.3 Whole-body cortisol levels (picograms, pg; nanograms, ng) of egg-sac fry (ESF; A), free-swimming fry (FSF; B), and young-of-the-year (YOY; C) of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*). Cortisol levels were measured either before (baseline; solid bars) or after (stressed; hatched bars) subjecting the offspring to 1 min of air exposure followed by a 5 min recovery period. From each nest, average cortisol content was quantified from groups of 12-30 ESF (n = 5 control baseline—cb, 5 control stressed—cs, 8 cortisol-treated baseline—tb, 9 cortisol-treated stressed—ts nests) and FSF (n = 5 cb, 9 cs, 8 tb, 8 ts nests), while YOY from each pond were processed individually (n = 38 cb, 20 cs, 34 tb, 11 ts individuals). Values are means + SEM. Treatments that do not share a letter are significantly different from one another (two-way ANOVA; see text for details); where no letters are present, no statistically significant differences were detected.

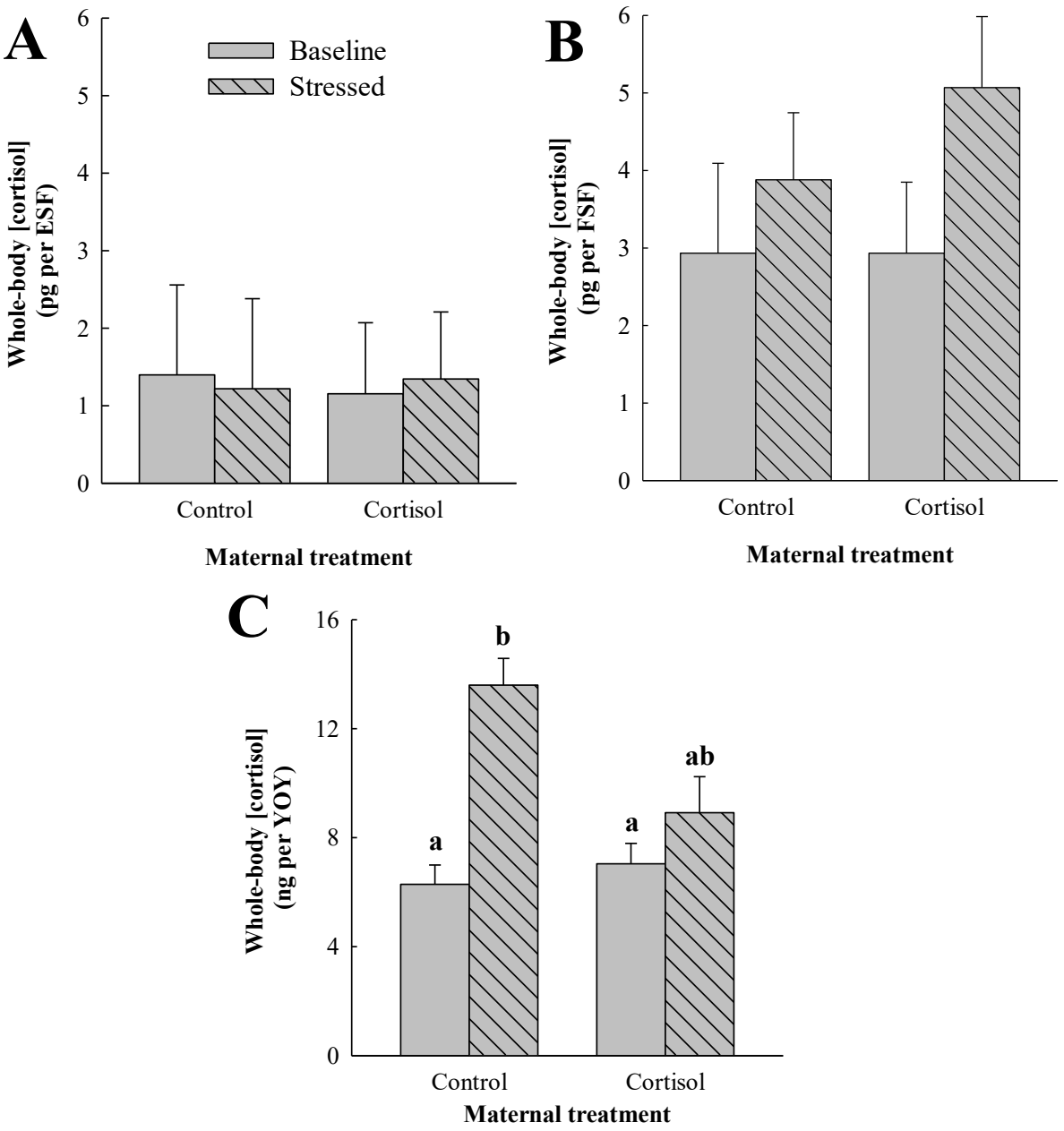
A
Baseline
Stressed
Whole-body [cortisol] (pg per ESF)
0
1
2
3
4
5
6
Control
Cortisol
Maternal treatment
B
Whole-body [cortisol] (pg per FSF)
0
1
2
3
4
5
6
Control
Cortisol
Maternal treatment
C
Whole-body [cortisol] (ng per YOY)
0
4
8
12
16
a
b
a
ab
Control
Cortisol
Maternal treatment

Figure 3.3

Figure 3.4 Latency for young-of-the-year (YOY) of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*) to emerge from a refuge into a novel environment (A), as well as the percentage of individuals that failed to emerge during a 15 min trial (i.e. % emergence failure; B). Values are means + SEM. An asterisk indicates a significant difference between maternal treatments (n = 29 YOY in each treatment; Student's *t*-test; see text for details).

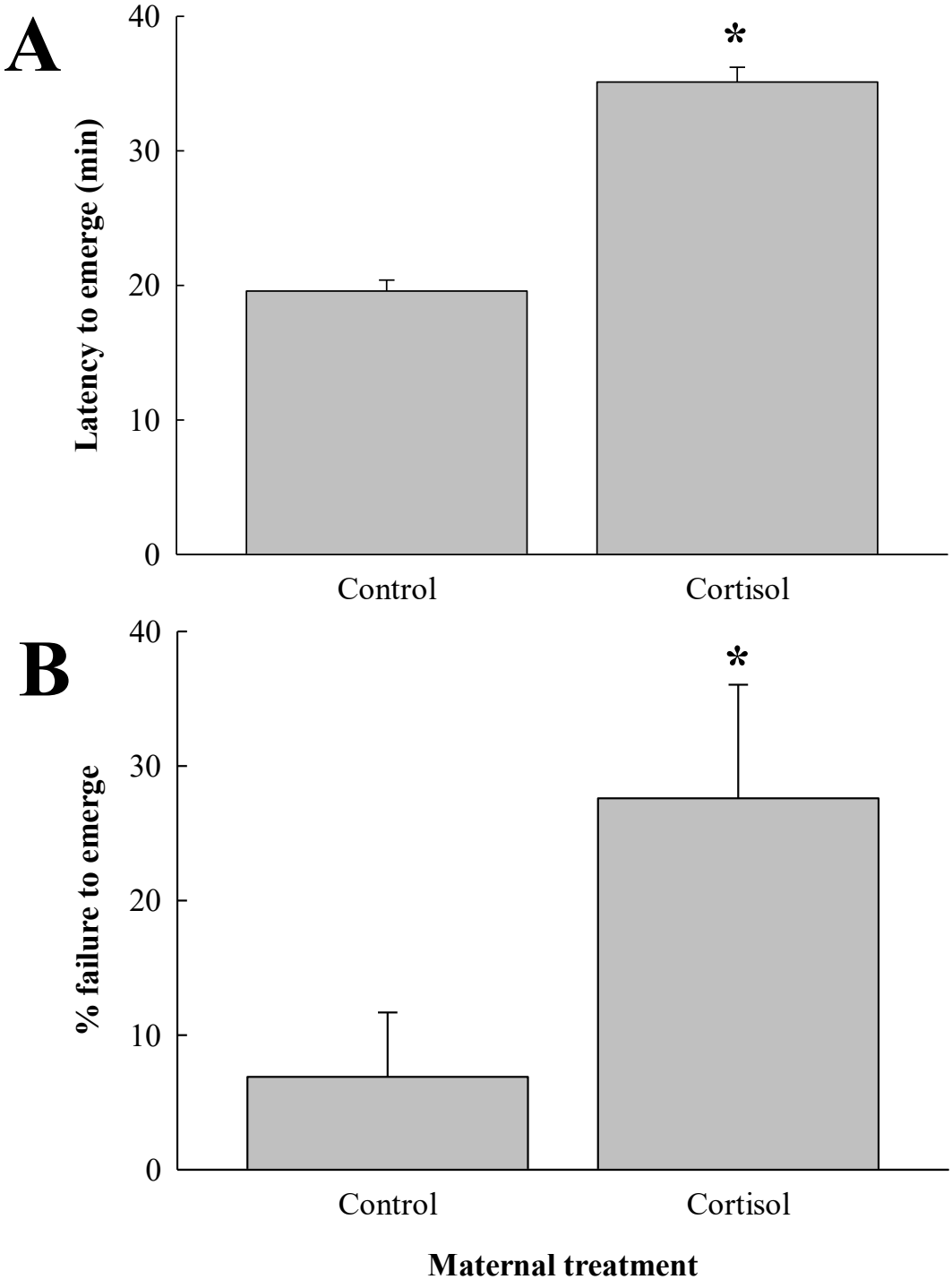
A
40
30
20
10
0
Latency to emerge (min)
*
Control
Cortisol
B
40
30
20
10
0
% failure to emerge
*
Control
Cortisol
Maternal treatment

Figure 3.4

Figure 3.5 Latency for young-of-the-year (YOY) of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*) to begin moving following visual exposure to an adult largemouth bass potential predator (A). The percentage of individuals that failed to move during a 10 min trial (i.e. % recovery failure; B) is also illustrated. Values are means + SEM. An asterisk represents a significant difference between maternal treatments (n = 29 YOY of control and n = 28 YOY of cortisol-treated females; Student's *t*-test; see text for details).

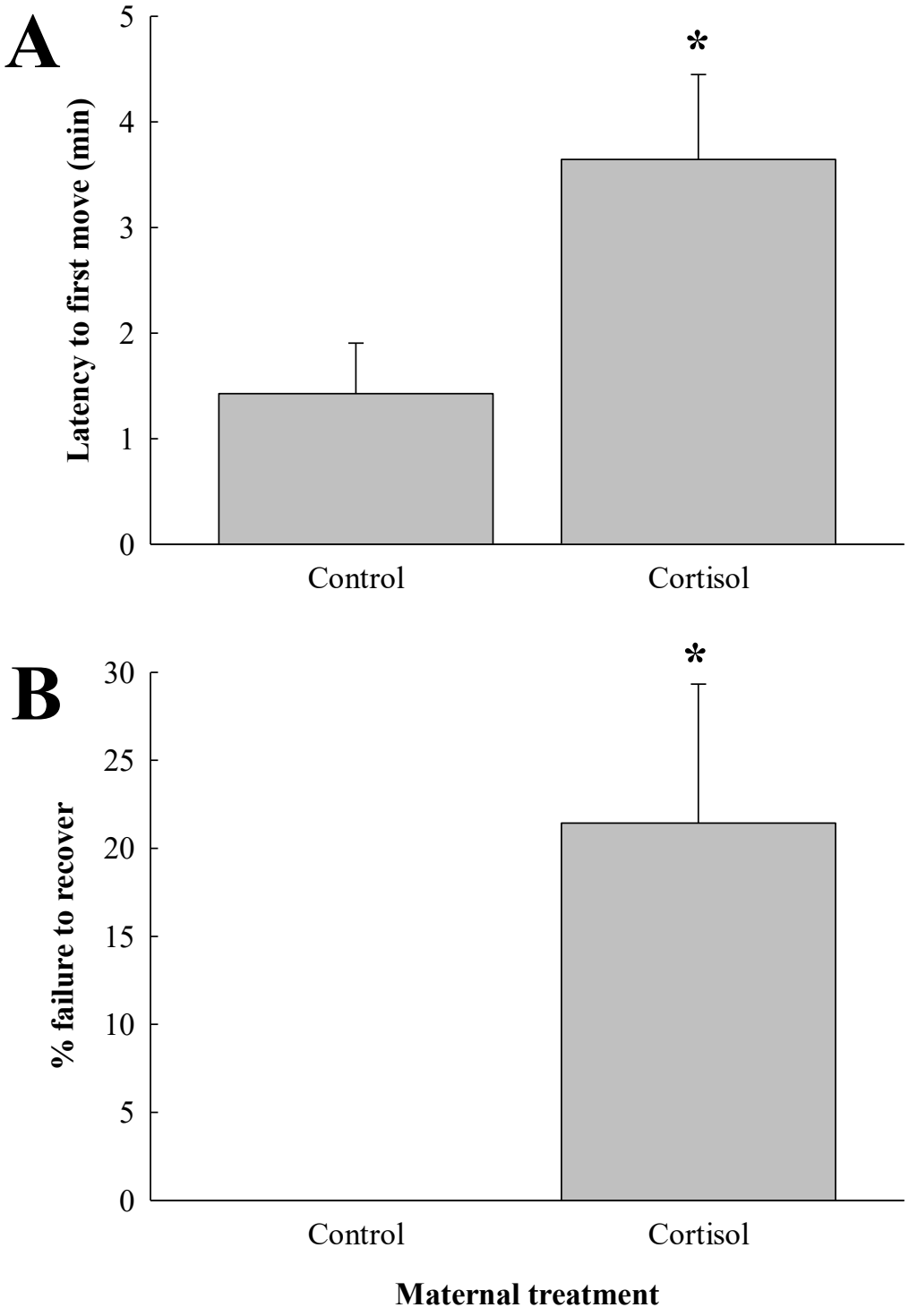
A
Latency to first move (min)
0
1
2
3
4
5
*
Control
Cortisol
B
% failure to recover
0
5
10
15
20
25
30
*
Control
Cortisol
Maternal treatment

Figure 3.5

Figure 3.6 The percentage of time free-swimming fry (FSF; A; n = 26 FSF of control and n = 35 FSF of cortisol-treated females) and young-of-the-year (YOY; B; n = 29 YOY of control and n = 24 YOY of cortisol-treated females) of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*) spent in the outer ring of the testing arena, thus exhibiting thigmotaxic anxiety, during a five min open-field test. Values are means + SEM. An asterisk indicates a significant difference between maternal treatments (Student's *t*-test; see text for details).

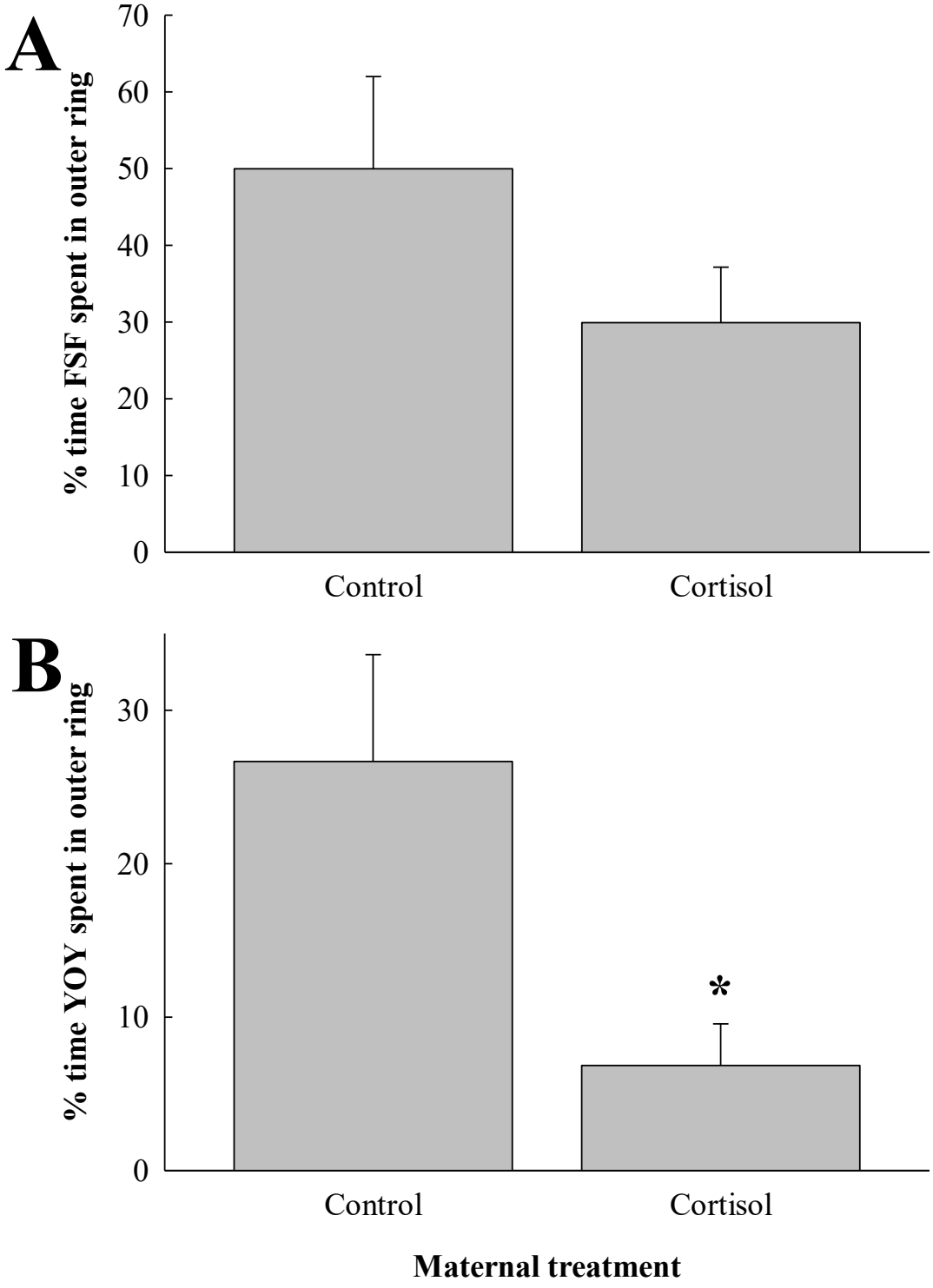
A
% time FSF spent in outer ring
70
60
50
40
30
20
10
0
Control
Cortisol
B
% time YOY spent in outer ring
30
20
10
0
*
Control
Cortisol
Maternal treatment

Figure 3.6

Table 3.1 Statistical results for analysis of the behavioural endpoints quantified during the open-field and black-white preference tests conducted on free-swimming fry (FSF) of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*).

Behaviour measured	Mean ± SEM		P-value
	Control	Cortisol-treated	
Open-field test [1]			
n = 26 FSF of control females and 35 FSF of cortisol-treated females			
# freezing bouts	9.36 ± 3.26	14.5 ± 3.3	0.103 *
% time spent active	11.6 ± 5.09	7.80 ± 2.53	0.302 *
Distance traveled (m)	4.08 ± 1.44	3.01 ± 0.65	0.299 *
Average velocity (cm s^{-1})	1.37 ± 1.20	0.992 ± 0.632	0.285 *
Black-white preference test [2]			
n = 29 FSF of control females and 40 FSF of cortisol-treated females			
% time spent in black half of test arena (scototaxic anxiety)	72.9 ± 5.9	61.2 ± 7.5	0.128 †

* ANOVA, nest included as random effect

† Student's *t*-test

Table 3.2 Statistical results for analysis of the behavioural endpoints quantified during open-field tests, black-white preference tests, and mirror tests of aggression conducted on young-of-the-year (YOY) of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*).

Behaviour measured	Mean ± SEM		P-value
	Control	Cortisol-treated	
Open-field test			
n = 29 YOY of control and 27 YOY of cortisol-treated females			
# freezing bouts	13.4 ± 1.9	11.0 ± 2.1	0.152 *
% time spent actively swimming	4.90 ± 1.1	5.00 ± 1.4	0.186 *
Distance traveled (m)	2.90 ± 0.31	2.44 ± 0.36	0.113 *
Average velocity (cm s^{-1})	0.969 ± 0.104	0.814 ± 0.119	0.116 *
Black-white preference test			
n = 30 YOY of control and 25 YOY of cortisol-treated females			
% time spent in black half (scototaxic anxiety)	90.8 ± 4.7	93.1 ± 4.6	0.367 †
Mirror test of aggression			
n = 30 YOY of control and 30 YOY of cortisol-treated females			
Latency to approach mirror (min)	6.93 ± 0.79	8.20 ± 1.12	0.390 *
Latency to begin aggressive behaviour/tail whipping (min)	12.2 ± 1.5	10.0 ± 1.2	0.228 *
# of bouts of tail whipping initiated	23.6 ± 2.6	21.1 ± 2.2	0.214 †
% of time spent engaged in tail whipping	1.23 ± 0.43	1.21 ± 0.94	0.495 Δ
% of time spent head-on with mirror	18.1 ± 3.1	16.3 ± 2.2	0.428 Δ

* Mann-Whitney U rank sum test
† Student's *t*-test
Δ ANOVA, pond included as random effect

3.5 DISCUSSION

The results of the present study support the hypothesis that administration of exogenous cortisol to wild, female largemouth bass prior to spawn, as a proxy for maternal stress, impacts offspring physiology and behaviour. Specifically, offspring of cortisol-treated females exhibited an attenuated cortisol response to an acute stressor together with behaviour suggestive of less boldness and less anxiety. These offspring effects were detected in the absence of a difference in embryo cortisol concentrations, prompting consideration of potential mechanisms other than increased deposition of maternal cortisol into the eggs. The observed effects of maternal cortisol treatment (as a proxy for maternal stress) on offspring will be discussed in the context of possible adaptive programming of offspring phenotype with respect to the hypothesis of transgenerational adaptive plasticity (Mousseau and Fox, 1998; reviewed by Green, 2008).

3.5.a Effects of exogenous cortisol administration on female spawning and embryo cortisol concentrations

In previous studies, stressor exposure in female teleost fish prior to spawn was reported to delay spawning (Mileva et al., 2011; Campbell et al., 1992; but see Contreras-Sanchez et al., 1998) and to reduce spawning success (Morehead et al., 2000). However, based on counts of males guarding nests with eggs, cortisol-treated largemouth bass females in the present study did not exhibit delayed spawning or lower spawning success. It should be noted that in 2014, all experimental fish, regardless of treatment group, began and completed spawning approximately two weeks later and earlier, respectively, than normal at the Sam Parr Biological Station; i.e. April 17th – May 2nd instead of the historical average of spawning occurring from the beginning

of April to mid-March (data not shown). The unusual spawning period in 2014 could be attributed to the unusually long period of consistent ice cover on the experimental ponds from January 2nd until March 6th in 2014; Sam Parr normally experiences multiple ice on-off events in the spring rather than one long period of ice cover. Alternatively, the delay in spawning may have been influenced by unusually heavy rainfall; Southern Illinois experienced several rainstorms in the spring of 2014, resulting in over twice as much precipitation in April 2014 than the historical average (225 mm vs 96 mm; USA Climate Data, April 2014). Regardless of the cause of the unusual spawning dates in 2014, the resultant delay between cortisol administration and spawning may have contributed to the apparent lack of effect of cortisol treatment on spawning date or breeding success.

The same factor, i.e. that spawning did not begin until 13 DPI and occurred as late as 28 DPI, also may have contributed to the lack of difference in embryo cortisol content between control and cortisol-treated females. O'Connor et al. (2013), using the same cortisol injection protocol as the present study, detected elevated ovarian cortisol concentrations in female bass at 9-13 DPI. Thus, exogenous cortisol in the cocoa butter implant may have been depleted by the time the developing eggs of the present study reached vitellogenesis, the phase of oogenesis during which cortisol (and other maternal egg provisions) are deposited into the egg yolk. A second possible explanation for the lack of difference in embryo cortisol content in the present study is maternal or embryonic buffering against maternal deposition of cortisol. Females may buffer eggs from hypercortisolism via the action of 11βHSD-2, the enzyme that converts cortisol to its inactive form cortisone. For example, zebrafish ovarian tissue treated with cortisol *in vitro* elevated ovarian follicle 11βHSD-2 transcript abundance 7-fold, potentially accounting for the

general lack of effect of maternal cortisol treatment on embryo cortisol content in this species, despite elevated maternal ovarian cortisol levels (Faught et al., 2016). Alternatively or additionally, offspring may self-protect from hypercortisolism by promoting efflux of cortisol using ATP-binding cassette (ABC) transporters, as proposed for three-spined stickleback embryos (Paitz et al., 2016). Clearly, these potential mechanisms require further investigation in largemouth bass. Although studies of embryos reared from cortisol-treated mothers or cortisol-treated eggs typically have reported elevated embryo cortisol levels (Sopinka, 2015; Burton et al., 2011; Eriksen et al., 2006; but see Faught et al., 2016), the absence of differences in embryo cortisol content in the present study is consistent with studies that utilized stressed females, which have reported no significant differences in cortisol levels between embryos of stressed and unstressed females (Jeffrey and Gilmour, 2016; Sopinka et al., 2014, but see Giesing et al., 2011; McCormick, 1998, 2006; Stratholt et al., 1997).

Despite the absence of elevated cortisol concentrations in embryos of cortisol-treated females, maternal cortisol treatment significantly affected offspring phenotype, suggesting that mechanisms other than embryo cortisol levels per se may mediate the effects of maternal stress on offspring. For example, maternal or embryonic buffering could be playing a role (Faught et al., 2016; Paitz et al., 2016), or altered maternal egg provisions other than cortisol (De Jesus and Hirano, 1992), or epigenetic effects on offspring gene expression (Szyf et al., 2005).

3.5.b Effects of maternal cortisol treatment on offspring growth

The present study found effects of maternal cortisol treatment only on offspring mass right after hatch, when early ESF of cortisol-treated females were greater in mass than offspring

of untreated control females. Although the absolute difference in average mass between the treatment groups was small (0.7 mg), the average mass of early ESF of cortisol-treated females (2.8 mg) was 33% higher than that of early ESF of control females (2.1 mg). No difference in body length was observed at this time point, suggesting that the mass difference reflected a difference in yolk volume, mass and/or composition. Separating yolk from larval body tissue would allow this possibility to be tested, but was not pursued in the current study which was conducted in the field. It is difficult to place this finding in the context of the existing literature for teleosts. First, the effect was transient, raising questions about its biological significance. Second, previous studies of the effects of maternal stress or cortisol exposure on offspring length and/or mass have reported variable results. Specifically, many studies reported reduced offspring length and mass (Burton et al., 2011; Contreras-Sanchez et al., 1998; Eriksen et al., 2006; Mileva et al., 2011; McCormick, 1998, 1999, 2006, 2009; Taylor et al., 2015), but others reported increases (Giesing et al., 2011; Mommer and Bell, 2014; Nesan and Vijayan, 2016; Sloman, 2010) or no difference between offspring of maternal treatment groups (Campbell et al., 1992, 1994; Sopinka et al., 2014; Stratholt et al., 1997). Species differences and/or differences in the type of maternal stressor or cortisol administration may account for at least some of this variability.

3.5.c Effects of maternal cortisol treatment on offspring stress-induced cortisol

The present study is, to our knowledge, the first to report baseline and stress-induced cortisol concentrations for largemouth bass fry. Maternal cortisol treatment did not have an effect on baseline cortisol concentrations of largemouth bass offspring at any of the three

developmental stages assessed. This finding is consistent with the majority of previous studies in free-swimming fish fry (Jeffrey and Gilmour, 2016; Mommer and Bell, 2013; Sopinka et al., 2016). Regardless of maternal treatment group, neither ESF nor FSF mounted a significant cortisol response to acute air exposure in the present study. Similarly to other teleosts species that have been examined (rainbow trout, *Oncorhynchus mykiss*, Auperin and Geslin, 2008; zebrafish, Alsop and Vijayan, 2008; Alderman and Bernier, 2009), the capacity to perceive external stressors and activate the HPI axis to generate a cortisol response appears during the transition from yolk reabsorption to exogenous feeding. By the YOY life-stage, a robust cortisol response was present and YOY of cortisol-treated females exhibited an attenuated cortisol response to air exposure compared to YOY of control females. This result is consistent with previous findings in other teleost species, which reported that offspring of stressed or cortisol-treated females exhibited an attenuated cortisol response to acute stress (Auperin and Geslin, 2008; Jeffrey and Gilmour, 2016; Sopinka et al., 2016). Similarly, cortisol-injected eggs yielded offspring with an attenuated cortisol response to an acute stressor (Nesan and Vijayan, 2016). If offspring of stressed females enter a 'stressful' environment, then an attenuated cortisol response may be adaptive according to the maternal match hypothesis of Love and Williams (2008), because it could alleviate some of the physiological costs associated with constant or repeated activation of the HPI axis.

3.5.d Effects of maternal cortisol treatment on offspring behaviour

In addition to the effect of maternal cortisol treatment on the cortisol response to an acute stressor in YOY, YOY of cortisol-treated females took longer to recover behaviourally from

acute stress. That is, YOY of cortisol-treated females exhibited a longer latency to begin moving after freezing in response to viewing a predator, and were significantly more likely to fail to recover than YOY of untreated control females. Put differently, maternal cortisol treatment resulted in offspring with a more persistent fear response. Additionally, YOY of cortisol-treated females displayed reduced boldness during the emergence test because they exhibited a longer latency to leave the refuge and explore the novel environment (and also failed to emerge more often during the behavioural trial). Together, these two results suggest that experimentally elevated maternal cortisol causes a more fearful, less bold behavioural phenotype in offspring.

Fear-related behaviours, such as freezing in the presence of a predator, might be expected to be exhibited in parallel with greater anxiety-related behaviour. However, increased anxiety (which is maladaptive; Perna et al., 2016) has been associated with impairment of fear responses that otherwise would have been adaptive to allow the threatened individual to escape from or cope with the imminent danger (zebrafish, Jesuthasan, 2012; rodents, Mongeau et al., 2003). As such, it is perhaps not surprising that the present study observed decreased anxiety-related behaviour (i.e. decreased thigmotaxic anxiety in the open-field test) in parallel with increased fear-related behaviour in YOY. This seemingly contradictory finding is perhaps more understandable given that the opposite trend (i.e. increased anxiety and decreased fear) was correlated with neural ageing in the form of increased rates of neurodegeneration and decreased neurogenesis (Perna et al., 2016). Thus, the results of the present study suggest an adaptive effect of experimentally elevated maternal cortisol on offspring behaviour. It should be noted, however, that the present experiment assessed multiple anxiety-related behaviours besides thigmotaxis, including freezing behaviour in the open-field test (different from the fear-related freezing behaviour in the predator exposure test) and scototaxic anxiety in the black-white

preference test. However, only thigmotaxic anxiety exhibited significant differences with maternal cortisol treatment. Additional work may be needed to optimize each anxiety test performed to avoid the influence of non-target behaviour, such as the natural aversion of larval fish to bright light (see Ch. 4).

3.5.e Conclusions

In summary, the treatment of wild, female largemouth bass with exogenous cortisol prior to spawn had long-lasting effects on offspring phenotype, including increased mass right after hatch, reduced boldness and anxiety-related behaviour but increased fear-related behaviour in YOY, and perhaps most strikingly, an attenuated cortisol response to an acute stressor in YOY. These results are consistent with the hypothesis of transgenerational adaptive plasticity (Mousseau and Fox, 1998; reviewed by Green, 2008), although further studies are needed to assess offspring traits directly related to fitness (e.g. over-winter survival, anti-predator behaviour, long-term survival and growth, reproductive success). It is noteworthy that aside from the impact on offspring mass right after hatch, the effects of maternal cortisol treatment did not manifest in offspring until later in development (up to 60 DPF), thus highlighting the importance of intergenerational studies to assess more than early embryonic or larval traits. Lastly, offspring effects of maternal cortisol treatment were observed in the present study even in the absence of differences in embryo cortisol concentrations, suggesting that maternal cortisol deposition into the egg is not the sole mediator of maternal stress effects on offspring phenotype. Other possible mechanisms, including epigenetic effects or altered egg provisioning of hormones and nutrients, warrant further investigation.

CHAPTER 4

GENERAL DISCUSSION

4.1 Summary and comparison of results

The present thesis tested the hypothesis that offspring phenotype is affected by maternal cortisol and social status in wild largemouth bass and domesticated zebrafish. Ch. 2 reported that, in zebrafish, maternal social interaction, as well as the resulting status of the interacting females can affect offspring phenotype, thus providing evidence in support of the hypothesis. However, contrary to our predictions, the effects of maternal social status on offspring were relatively minor and not unique to offspring of subordinate females. Instead, offspring of dominant females exhibited increased boldness and decreased anxiety-related behaviour compared to offspring of sham and subordinate females. Arguably, the most striking results of Ch. 2 were the effects of maternal social interactions in general, regardless of the resulting status of the females. That is, offspring of females that engaged in social interactions exhibited significantly better survival at 1 DPF, longer bouts of freezing in response to bright light at 6 DPF, and decreased baseline whole-body cortisol at 0 and 30 DPF. Interestingly, increased boldness was observed in offspring of dominant females and offspring of females that were not treated with exogenous cortisol also tended to exhibit greater boldness, suggesting that effects of maternal dominance on offspring behaviour may have been mediated at least in part by (relatively low) maternal cortisol, thus providing evidence in support of maternal cortisol as a mediator of effects of maternal social status. Importantly, effects of maternal social interaction and/or status on offspring generally occurred in the absence of elevations in embryo cortisol content. Additionally, the lack of similarities between offspring of cortisol-treated females and offspring of females that engaged in social interactions suggest that maternal cortisol likely is not the only factor mediating effects of maternal social experience on offspring. Possible mediating

factors worthy of further investigation include the physiological consequences of the constant exercise experienced by females engaging in aggressive dyadic social interactions.

In contrast, Ch. 3 reported significant effects of experimentally elevated maternal cortisol on offspring mass, cortisol responses to stress, and behaviour in wild largemouth bass, thus providing evidence in support of the role of cortisol in mediating effects of maternal stress on offspring. Specifically, offspring of cortisol-treated females exhibited greater mass right after hatch, a significant attenuation of stress-induced whole-body cortisol in response to acute air exposure, decreased boldness, and decreased thigmotaxic anxiety. Again, all effects of maternal cortisol exposure on offspring occurred in the absence of differences in embryo cortisol content.

A comparison of the results reported in Ch. 2 and 3 revealed two noteworthy similarities as well as several differences. First, in both studies, offspring of cortisol-treated females tended to take longer to explore novel areas, and thus exhibited decreased boldness. This observation raises the question of what mechanism could be linking elevated maternal cortisol to altered offspring behaviour several days after hatch. Contrary to our prediction, the results of the present thesis suggest that the mechanism driving offspring effects of maternal stress was not increased maternal deposition of cortisol into egg yolks causing downstream alteration of early development post-fertilization. This conclusion stems from the observation in both studies of the present thesis that embryos of socially-stressed or cortisol-treated females did not contain significantly higher levels of maternally-deposited cortisol. This finding is consistent with those of other studies in teleosts where no difference in the cortisol content of embryos reared from stressed versus unstressed females was detected, including dyadic maternal social hierarchy formation in zebrafish (Jeffrey and Gilmour, 2016), repeated chasing in sockeye salmon, *Oncorhynchus nerka* (Sopinka et al., 2014), cortisol consumption in zebrafish (Faught et al.,

2016), and chasing and netting in the cichlid *Neolamprolous pulcher* (Mileva et al., 2011). At the same time, however, some studies have detected differences in embryo cortisol (e.g. Giesing et al., 2011; McCormick, 1998, 2006; Stratholt et al., 1997). As such, identification of the mechanism(s) responsible for mediating effects of maternal stress on offspring requires further investigation. One possible explanation for the lack of increase in embryonic cortisol despite elevated maternal cortisol is maternal buffering against embryonic hypercortisolism via increased activity of ovarian 11βHSD-2, which converts active cortisol to inactive cortisone, as suggested by Faught et al. (2016). These authors noted that 11βHSD-2 expression in zebrafish ovarian tissue *in vitro* increased seven-fold following treatment with cortisol.

The results reported in the present thesis also revealed differences in the phenotypic traits of offspring of cortisol-treated or stressed zebrafish versus cortisol-treated largemouth bass. For example, offspring of cortisol-treated bass but not zebrafish exhibited significantly reduced stress-induced cortisol levels in response to acute air exposure. However, zebrafish females that participated in social interactions produced offspring that exhibited decreased baseline cortisol levels at some time points. The cause of these differences remains unclear. Although the two studies had a similar basic experimental design, they were conducted on different species (zebrafish vs bass) of different orders (Cypriniformes vs Perciformes) with different reproductive styles (continuous vs synchronous) in different settings (laboratory vs wild). In addition, the dose, timing and site of injection of cortisol, as well as the delivery medium, differed between the two studies, likely resulting in different patterns of cortisol elevation in the females prior to spawn (reviewed by Sopinka et al., 2015). Specifically, zebrafish females were given a quick-release intramuscular injection of cortisol in saline two days prior to reproduction, a time when vitellogenesis is likely complete, whereas largemouth bass females were given a slow-release

intraperitoneal injection of cortisol emulsified in cocoa butter ~2-3 weeks prior to spawning. Comparisons between the studies of the present thesis support the need for laboratory studies to explore mechanisms in a setting that allows a high level of control over variables, while at the same time highlighting the need for research of ecologically-relevant questions to be conducted in wild species in their natural environment.

4.2 Potential impact of observed effects of maternal social interactions and cortisol treatment on population dynamics

In a maternal match situation ("Maternal Match Hypothesis" reviewed by Breuner, 2008; Love et al., 2005, 2013), both the maternal and offspring environment have a high probably of frequent/constant exposure to stressors. The results of the present thesis provide some support for the notion that females exposed to social interactions (zebrafish) or exogenous cortisol (bass) produce offspring with traits that may increase chances of offspring success in an equally stressful environment. Thus, the results of the present thesis provide some support for the hypothesis of transgenerational adaptive plasticity (Mousseau and Fox, 1998; Green, 2008). That is, as suggested by Love et al. (2013), effects of maternal stress may not be simple unavoidable costs of altered development as a result of altered maternal ovarian condition, but instead may be adaptive responses to prepare offspring to cope in a matched environment. For example, the observation of decreased offspring cortisol concentrations (baseline in zebrafish or stress-induced in bass) may reduce the energetic cost associated with frequently elevating cortisol concentrations in a maternally-matched stressful offspring environment. Similarly, decreased boldness may prove beneficial in an environment laden with predators. Together, the results suggest greater fitness potential in offspring of females that underwent social interactions

or cortisol treatment if those offspring experience a stressful environment. It must be considered, however, that in a maternal mismatch situation, the effects of maternal social interaction and cortisol treatment observed in the present thesis may negatively affect offspring success.

Future studies should investigate whether maternal social interactions or cortisol treatment impact fitness-relevant traits in offspring, such as lifetime survival and reproductive success. If future studies reveal significant effects of maternal stress on fitness-relevant traits, then aquaculture and conservation managers will need to become more attentive to not only the effects of stress on maternal survival, but also the potential impact of maternal stress and social interactions on population dynamics. For example, effects on offspring survival may affect population numbers potentially leading to downstream effects on predator and/or prey ecology. Also, if an entire generation produced from stressed females exhibits decreased boldness as the results of the present thesis suggest, this altered behaviour could affect future reproductive success depending on mate and predator abundance.

Alongside the potential ecological impact, the results of the present thesis also warn of a potential economical and/or cultural impact. The popularity of largemouth bass as a gamefish can be attributed in part to the species' notorious boldness in taking bait and aggression once hooked. The present thesis observed a significant decrease in boldness in offspring of females with elevated cortisol. Härkönen et al. (2016) reported that fish with a high catchability tend to be those that are quicker to explore a novel environment and a novel object (such a bait or a lure), thus exhibiting high boldness. Consequently, if a stressful bass environment leads to a decrease in the boldness of subsequent generations, then the appeal of bass as a gamefish may decrease with time, and the multibillion dollar fishing industry (Williamson, 2016) may suffer.

4.3 Experimental critique

4.3.a Effect of light intensity on larval behaviour

The present thesis assessed activity of larval zebrafish and largemouth bass by conducting an open-field test with automatic tracking. The tracking software required lighting conditions that achieved sharp contrast between the light background and the fish to correctly identify the fish as the object of interest. In 6 DPF zebrafish, ambient lighting was not sufficient because apart from their two dark eyes, larval bodies are translucent. However, the addition of two fluorescent lamps achieved the necessary contrast. As previously discussed, this environment elicited a larval fear response to the abrupt exposure to bright light. Largemouth bass offspring were relatively large and dark, and ambient light allowed for the correct identification of the subject. Although the bright lighting conditions affected larval zebrafish fear responses during the open-field tests conducted in the present thesis, thigmotaxic anxiety probably was not affected because the testing chamber was equally brightly lit throughout, therefore not likely affecting the tendency of larvae to self-situate along the outer walls.

Automatic tracking of offspring movement was not an option for the black-white preference tests conducted in the present thesis because both zebrafish and largemouth bass larvae were not detectable when they were situated in the black half of the tank, regardless of lighting conditions. To maximize the difference in brightness between the dark and light halves of the testing arena, the light half was both white in background colour and brightly lit with a fluorescent lamp directly overhead. Unfortunately, these lighting conditions meant that offspring spent almost the entire trial (i.e. an average of 99.7% of the 5 min trial) in the dark half of the arena. Any potential effects of maternal treatment on offspring scototaxis were likely masked by this exaggerated scototaxic anxiety. Therefore, the results of the light-dark preference tests (in

both studies) should at best be interpreted with caution. Future studies should conduct anxiety tests of larval fish in dimly lit conditions to minimize fear responses and to amplify any subtle differences in innate anxiety-related behaviour between treatment groups. Apart from illumination, all other potential sources of sampling error associated with the automatic tracking of larval zebrafish movement outlined in a review by Martineau and Mourrain (2013) were accounted for within the algorithms of the tracking software used in the present thesis.

4.3.b Ambiguity of measuring behaviour

Behaviours are context-dependent and not mutually exclusive. For this reason, it is important for future behavioural studies to be aware of the context in which a behaviour endpoint is measured. For example, different types of behaviours can be measured by one action if assessed in different contexts; e.g. freezing can be a measure of anxiety in the absence of a stressor or fear in the presence of a stressor (e.g. open-field versus the predator exposure test, respectively). In addition, a single action in a particular context can assess multiple types of behaviours. For example, the observed action of freezing following predator exposure may reflect not only boldness, but also fearfulness and/or exploratory behaviour. Conversely, a single behaviour can be measured by many different actions; e.g. the trait of boldness was assessed in the present thesis by both the emergence and novel tank diving tests by measuring the latency of offspring to enter a novel area via horizontal and vertical exploration, respectively. Conclusions can be more difficult to draw from data in which different tests assessing the same type of behaviour (e.g. boldness) produce conflicting results. However, discrepancies highlight the importance of assessing a single behaviour in multiple contexts to achieve a broad understanding of its scope and how it is affected by the independent variable in question—in this case, maternal stress.

4.3.c Potential paternal effect

In both studies of the present thesis, it was not logistically practical to completely control for variation associated with paternal identity. Hanson and Cooke (2009) reported that in smallmouth bass, a species in which the male parent provides sole parental care to the brood (as in largemouth bass), female reproductive investment depended on male body length and "stoutness". A similar trend was reported in zebrafish, a teleost species that does not engage in parental care and thus exhibits a "resource free mating system" (Uusi-Heikkilä et al., 2012); female zebrafish consistently exhibited greater reproductive investment when breeding with larger males compared to smaller males (Uusi-Heikkilä et al., 2012). Owing to the potential effect of male size on offspring clutch size and quality, the males with which the experimental females were allowed to breed in the present thesis were therefore equally distributed by mass and length between maternal treatment groups. Male zebrafish assigned to each treatment group were randomly assigned to a breeding box containing a single experimental female, whereas groups of size-matched male bass were distributed equally into the six experimental ponds and left to breed naturally with one or more of the females in the pond.

Although the present thesis controlled for the potential effect of paternal size on female reproductive investment, the experimental design could not account for a potential effect of differential paternal reproductive investment in response to female condition. Possible examples include altered courtship behaviour or strength of intrasexual competition, or altered investment in parental care depending on the condition of the female parent (reviewed by Edward and Chapman, 2011). There is growing evidence of sexual selection by females (when fertile) for low GC levels in potential male mates (reviewed by Moore, 2012; humans, *Homo sapiens*,

Moore et al., 2011; zebra finches, *Tynopygea guttatta*, Roberts et al., 2007; great plain toads, *Bufo cognatus*, Leary et al., 2004), but literature exploring the reverse is lacking.

4.4 Future directions

4.4.a Further exploration of the effects of maternal stress and cortisol treatment on offspring

The results of the present spur new questions. First, in addition to delving deeper into the mechanisms underlying effects of maternal stress on offspring phenotype, future studies should explore whether effects persist or change into adulthood or across multiple generations. Second, whether maternal social interaction or cortisol exposure increases offspring fitness in a stressful environment, as the results of the present study suggested, also warrants further investigation. Fitness-relevant traits worthy of testing might include offspring lifetime survival, offspring survival in the presence of predators, as well as reproductive success. The observation that offspring of dominant females exhibited increased boldness and reduced geotaxic anxiety in zebrafish raises the question of whether social status can be inherited by offspring. Effects of maternal stress on offspring sex-ratio could also be explored. According to the sex-allocation theory, stressed females should theoretically invest more in offspring of the less expensive sex (Charnov, 1982). In teleosts, masculinization has been reported in broods exposed to environment stressors or exogenous cortisol during early development (Hattori et al., 2009; Hayashi et al., 2010; Yamaguchi et al., 2010).

4.4.b Could paternal stress affect offspring phenotype in teleosts?

The present thesis assessed the effects of maternal stress and cortisol treatment on offspring phenotype, but reproduction requires the investment of both females and males, so an

obvious next question is whether paternal stress can directly affect offspring phenotype. Previous studies in rats have reported effects of paternal stress prior to fertilization on offspring (Harker, 2013; Rodgers et al., 2013), and research in teleosts has reported effects of paternal stress post-fertilization via altered paternal care (Dey et al., 2010; McGhee and Bell, 2014; O'Connor et al., 2009; Stein and Bell, 2014). Interestingly, the reported effects on offspring of pre- and post-fertilization paternal stress show similarities to the effects on offspring of maternal cortisol exposure observed in the bass study of the present thesis.

A recent search of the literature did not reveal studies that have explored whether pre-spawn paternal stress affects offspring phenotype in teleosts, but this question is worthy of investigation because research in mammals has reported effects of paternal stress prior to fertilization on offspring phenotype, likely via effects on sperm (Harker, 2013; Rodgers et al., 2013). Male rats stressed prior to breeding produced sperm with altered microRNA proportions (Rodgers et al., 2013), and the offspring of the stressed male rats in turn exhibited brain location-dependent changes in DNA methylation and transcription, attenuated HPA axis responsiveness to acute stress, and decreased thigmotaxic anxiety (Harker, 2013; Rodgers et al., 2013). Interestingly, attenuated stress-induced GC levels and decreased thigmotaxic anxiety in offspring of cortisol-treated female bass were observed in the present thesis, suggesting common effects of parental exposure to stress/cortisol prior to fertilization on offspring phenotype.

Many teleost species exhibit paternal care, including largemouth bass, and studies have reported that cortisol-treated male bass abandoned their broods at a higher rate than untreated males (Dey et al., 2010; O'Connor et al., 2009). A negative effect of paternal stress on investment in brood care could in turn diminish offspring fitness. For example, three-spined stickleback offspring that lost their parental male exhibited increased anxiety and, as a result,

were captured sooner when faced with a predator (McGhee and Bell, 2014). Adult offspring of male sticklebacks exposed to predation risk during the period of parental care also exhibited reduced size and body condition, as well as an increased freezing response to predator exposure (Stein and Bell, 2014). Again, these observed effects of paternal stress post-fertilization mirror the behavioural effects of maternal cortisol exposure observed in bass in the present thesis; that is, decreased boldness and anxiety, as well as increased fear responses. Clearly, further investigation of effects of paternal stress on offspring is warranted.

4.5 Conclusions

The results of the present thesis provide evidence in support of the general hypothesis that maternal exposure to stress and exogenous cortisol affect offspring phenotype in domesticated zebrafish and wild largemouth bass. However, increased maternal deposition of cortisol into eggs is not likely the factor mediating these effects because effects on offspring were observed in the absence of elevations in embryo cortisol content. In the context of the hypothesis of transgenerational adaptive plasticity, the results suggest that female zebrafish who engaged in social interactions, regardless of their resulting social status, and largemouth bass females exposed to exogenous cortisol prior to fertilization may prime offspring with traits that may confer fitness benefits in an offspring environment that is matched in stressor intensity to the maternal environment.

APPENDICES

Appendix A:

Examples of the how variation in different portions of the experimental design (i.e. "source of variation") of maternal stress studies results in different effects on offspring characteristics even within offspring of stressed females of the same species.

Source of variation	Forms of differing factor	Offspring effect	Species	References
Type of stressor	Chasing vs. Intraspecific competition	Survival (ND vs. ↓)	Sockeye salmon (*Oncorhynchus nerka*)	Sopinka et al., 2014 vs. Essington et al., 2000
Duration of stressor exposure	Repeated acute (chasing) vs. single chronic (migration) exercise	Embryo size (diameter) (ND vs. ↓)	Sockeye salmon (*Oncorhynchus nerka*)	Sopinka et al., 2014 vs. Taylor et al., 2015
Phase of oogenesis during exposure	Early vs. late vitellogenesis	Egg size (↓ vs. ND)	Rainbow trout (*Oncorhynchus mykiss*)	Contreras-Sanchez et al., 1998
Location/ environment	Natural river vs. man-made channel	Body morphology (smaller fins and robust body vs. larger fins and streamlined body)	Sockeye salmon (*Oncorhynchus nerka*)	Sopinka et al., 2014
Offspring age	Before vs. after hatching	Survival (↓ pre-hatch vs. ↑ post-hatch)	Tropical damselfish (*Pomacentrus amboinensis*)	Gagliano and McCormick, 2009

ND **no difference**

Appendix B:

Points system used to assign social status to size-matched adult zebrafish (*Danio rerio*) females confined in pairs following hierarchy formation. A higher score is reflective of dominant behaviour.

Behaviour	Description	Score
Position	Bottom/top/corner – immobile	0
	Bottom/top/corner – little movement	5
	Patrolling entire tank	10
Chasing/biting	None	0
	1-5	1
	6-10	2
	11-15	3
	16-20	4
	>20	5
Retreats	>10	0
	6-10	1
	1-5	2
	None	3
Feeding	Did not feed first	0
	Fed first	1

Appendix C:

Protocol used to extract behaviour data from video recordings (and remove outlier data points from tracking output) for all behavioural endpoints assessed during the open-field test 6 DPF zebrafish (*Danio rerio*) larvae.

1. The exact time (s) within the video to begin tracking fish movement following the 2 min acclimation and removing the starting chamber was determined.

2. Fish movement using custom-made tracking software, written in Python by Aaron Shifman (script not included due to length) was recorded. The tracking output (i.e. a series of approximately 9000 X-Y coordinates for each 5 min trial) was saved in Excel.

    *** Videos that could not be tracked with a sufficient degree of accuracy using the program written in Python were discarded. In total, 78 of 140 videos (i.e. 56%) were discarded. The remaining 62 videos had an average tracking efficiency of 93%. Tracking "inaccuracies" occurred when the program incorrectly identified the location of the test subject (i.e. gave the coordinates of a glare in the corner of the tank or a ripple in the water, etc.). Each of the remaining 62 videos was manually refined to identify and delete these false coordinates. The time between each data point was $1/30^{th}$ s, so an easily identified outlier/false coordinate (or a set of consecutive false data points) was defined as a coordinate that had a distance travelled of more than 1 cm since the coordinate of the previous frame. This time-consuming but necessary step increased the tracking efficiency from 93% to virtually 100%.

3. Determination of pixels to cm scale: A scale (# pixels per cm) was determined by first taking a screenshot of one video frame using VideoPad Video Editor. The JPEG image was scaled in ImageJ (Abramoff et al., 2004) by measuring the length (in pixels) of the known tank diameter. For example, a round test tank with a diameter of 10 cm measured as 1000 pixels in ImageJ would yield a scale of 100 pixels per cm.

4. Determination of the percentage of time spent in outer third of test arena:

    a. The length (pixels) of the radius from the center point to the edge of the water in the tank was recorded in ImageJ during step 3.

    b. The open-field test tank contains a visible centre point, so the X-Y coordinate (pixels) of the centre of the tank was recorded in ImageJ after the scale was set.

    c. The length of the radius (pixels) from the center point to the outer edge of each ringed area was determined by dividing the length (pixels) of the radius determined in step 4a, and dividing it by three (width of a single ringed area = X), and then performing the following calculations in Excel:

        - Radius to the edge of the center circle = X
        - Middle ring = X*2
        - Outer ring = X*3

    d. A MATLAB script was written (with the help of Benoit Tremblay) to determine the percentage of time the fish spent in each of the three (ring) areas of the tank around the recorded centerpoint. It adapted a published MATLAB function that determines the

number of points found within a circle of a set cut-off radius around a set center point (Anandatheertha, 2012). See Appendix D for the MATLAB script.

5. Determination of other behavioural endpoints in Excel:

   a. Total distance travelled: For each frame, the distance (pixels) travelled by the fish (i.e. distance between previous frame's X-Y coordinate and present X-Y coordinate) was calculated using the Pythagorean Theorem. This step was repeated for each of the 9000 frames, and each distance between coordinates was then converted to true distances (cm) using the previously determined scale for that particular video recording (see Eq. 1). Finally, the total distance traveled by the fish during the five min open-field trial was defined as the sum of all the distances travelled during the trial, and was determined using the following Excel function:

      =SUM("entire distance column")

      Eq. 1: $\frac{(X_2-X_1)^2+(Y_2-Y_1)^2}{\text{Scale (i.e. } {}^{\#\text{pixels}}/_{\text{cm}})} = \text{Total distance travelled (cm) during a single frame}$

   b. Percentage of time spent actively swimming:

      i. For each frame, the fish was deemed as immobile if the distance travelled within that frame was less than 0.1 cm using the following function to assign a score of 1 to all fish that were "immobile":

         =IF("distance for the frame"<0.1,1,0)

ii. The total number of video frames during which the fish was immobile was calculated by taking the "=SUM(__)" of all cells/coordinates obtained in step 5.b.i.

iii. The percentage of time spent active was then calculated using the following equation:

$$1 - \frac{\text{Total \# rows or frames during which the fish was "immobile"}}{\text{9000 (i.e. Total \# of frames in the video)}} * 100\%$$

c. Average swimming velocity:

i. Calculation of instantaneous velocity at each frame: The small distance between each set of two X-Y coordinates represents the distance traveled by the fish within one frame, which is $1/30^{th}$ of a s, so the instantaneous velocity (cm s^{-1}) of the fish at each frame was determined by dividing the true (cm) distance travelled by $1/30^{th}$ s (0.0333 s) using the following equation:

$$\frac{\text{Distance (cm)traveled during a single video frame}}{{}^{1}/_{\text{30th}}\,\text{(s)}}$$

= instantaneous velocity during a single video frame (cm s^{-1})

ii. The average velocity during the open-field trial was then determined by calculating the mean of the instantaneous velocities for all frames using the following Excel function:

=AVERAGE("entire instantaneous velocity column")

d. Number of bouts of activity: A single bout of activity was defined as one or more consecutive frames during which a fish was active. To quantify the number of these occurrences, a novel Excel Macros script was written by adapting the existing "COUNTIFS" function in Excel. See Appendix E for the Excel macros script. The number of bouts of activity during a whole video trial was determined by counting the instances that the immobility score was "0" (i.e. fish was active) for at least 1 frame, using the following Excel function:

=COUNTINSTANCESGREATERTHAN("entire immobility column",0,1)

Appendix D:

MATLAB script used to determine the percentage of time a fish spent in each of the three rings of the behavioural chamber during a five min open-field test. Input that varies with each video trial's data (i.e. X-Y coordinates per frame) is italicized.

```
clear
num = xlsread('Bass_fry_OF.xls','30_1_cm');
X=num(:,1);
Y=num(:,2);
radius = [3.3,6.6,10,20];
center = [23.57,15.36];
count=0;
count1=0;
count2=0;
count3=0;
count4=0;

for frames = 1:size(num,1)
   for radiusct = 1:size(radius,2)

      cutoffradius = radius(radiusct);

      [points]=pointsincircle({X(frames,1) Y(frames,1)},cutoffradius,center);

      points1=points.out{1}(:);
      if isempty(points1)
         points1=0;
      else
         points1=1;
      end
      count=count+points1;
   end

   %% This will count the presence of the fish in each radius
   if count==1
      count1=count1+1; %%%% outside largest radius
   elseif count==2
      count2=count2+1; %%% within largest radius
```

```
    elseif count==3
        count3=count3+1; %% within 2nd ring
    elseif count==4
        count4=count4+1; % withing smallest ring
    end
    count=0;
end

disp(['Outside largest ring count = ' num2str(count1) '  /  % of time : '
num2str(count1/size(X,1)*100) '%']);
disp(['Largest ring count = ' num2str(count2) '  /  % of time : ' num2str(count2/size(X,1)*100)
'%']);
disp(['Middle ring count = ' num2str(count3) '  /  % of time : ' num2str(count3/size(X,1)*100)
'%']);
disp(['Smallest ring count = ' num2str(count4) '  /  % of time : ' num2str(count4/size(X,1)*100)
'%']);
```

Appendix E:

Excel macros used to calculate the number of bouts of active swimming performed in the open-field test; i.e. the number of instances that the distance travelled by a fish within a frame was at least 0.1 cm (any distance less than 1 mm was defined as "immobile" likely due to tracking error) for at least 1 frame. A description of the use of the created function follows the macros script. Input that varies with each video trial's data is italicized.

```
Function CountInstancesGreaterThan(rng As Range, NumberToCheck As Long, RepeatTimes
As Long)
   Dim strSequence As String
   Dim n As Long, ctr As Long

   For n = 1 To rng.Cells.Count + 1
      If rng(n, 1) = NumberToCheck Then
         strSequence = strSequence & rng(n, 1)
      Else
         If Len(strSequence) >= RepeatTimes Then
            ctr = ctr + 1
         End If
         strSequence = ""
      End If
   Next

   CountInstancesGreaterThan = ctr

End Function


Sub Freezes()

End Sub
```

To use the function on the Excel worksheet interface, insert (variable input in italics): "=CountInstancesGreaterThan(*range of cells indicating whether the fish was active (0) or immobile (1)*, 0, 1)"

Appendix F:

Protocol used to extract behaviour data from video recordings of largemouth bass (*Micropterus salmoides*) fry and YOY during an open-field test.

1. Prepare video for automatic tracking:

    a. Convert video format from MTS to MP4 using Aiseesoft MTS Video Converter.

    b. Crop MP4 video so that it begins right after the acclimation chamber is removed and is exactly five min in length using VideoPad Video Editor.

2. Use IdTracker (Pérez-Escudero et al., 2014) to track the spatial location of the fish within each frame of the 5 min video recording. The output of IdTracker is one set of X and Y coordinates per video frame. Each video was recorded at 30 fps, so the output contained ~9000 location coordinates in pixels.

3. Determination of pixels to cm scale: A scale (# pixels per cm) was determined by first taking a screenshot of one video frame using VideoPad Video Editor, and then determining the scale by loading the JPEG image into ImageJ (Abramoff et al., 2004) and setting the 'scale' by measuring the length (in pixels) of the known tank diameter. For example, if a round test tank had a diameter of 10 cm and the diameter was measured as 1000 pixels long in ImageJ, then the scale would be calculated as 100 pixels per cm.

4. Determination of the percentage of time spent in outer third of test arena:

    a. The length (pixels) of the radius from the center point to the edge of the water in the tank was recorded in ImageJ during step 3.

b. The open-field test tank contains a visible centre point, so the X-Y coordinate (pixels) of the centre of the tank was recorded in ImageJ after the scale was set.

c. The length of the radius (pixels) from the center point to the outer edge of each ringed area was determined by dividing the length (pixels) of the radius determined in step 4a, and dividing it by three (width of a single ringed area = X), and then performing the following calculations in Excel:

    i. Radius to the edge of the center circle = X

    ii. Middle ring = X*2

    iii. Outer ring = X*3

d. A MATLAB script was written (with the help of Benoit Tremblay) to determine the percentage of time the fish spent in each of the three (ring) areas of the tank around the recorded centerpoint. It adapted a published MATLAB function that determines the number of points found within a circle of a set cut-off radius around a set center point (Anandatheertha, 2012). See Appendix D for the MATLAB script.

5. Determination of other behavioural endpoints in Excel:

    a. Total distance travelled: For each frame, the distance (pixels) travelled by the fish (i.e. distance between previous frame's X-Y coordinate and present X-Y coordinate) was calculated using the Pythagorean Theorem. This step was repeated for each of the 9000 frames, and each distance between coordinates was then converted to true distances (cm) using the previously determined scale for that particular video

recording (see Eq. 1). Finally, the total distance traveled by the fish during the five min open-field trial was defined as the sum of all the distances travelled during the trial while the fish was not immobile (see step 5b), and was determined using the following Excel function:

=SUMIF("entire immobility column",1,"entire distance column")

Eq. 1: $\frac{(X_2-X_1)^2+(Y_2-Y_1)^2}{\text{Scale (i.e. } {}^{\#\text{pixels}}/_{\text{cm}})} = \text{Total distance travelled (cm) during a single frame}$

b. Percentage of time spent actively swimming:

   i. For each frame, the fish was deemed as immobile if the distance travelled within that frame was less than 0.1 cm using the following function to assign a score of 1 to all fish that were "immobile":

      =IF("distance for the frame"<0.1,1,0)

   ii. The total number of video frames during which the fish was immobile was calculated by taking the "=SUM(__)" of all cells/coordinates obtained in step 5.b.i.

   iii. The percentage of time spent active was then calculated using the following equation: $1 - \frac{\text{Total \# rows or frames during which the fish was "immobile"}}{\text{9000 (i.e. Total \# of frames in the video)}} * 100\%$

c. Average swimming velocity:

   i. Calculation of instantaneous velocity at each frame: The small distance between each set of two X-Y coordinates represents the distance traveled by the fish

within one frame, which is $1/30^{th}$ of a s, so the instantaneous velocity (cm s^{-1}) of the fish at each frame was determined by dividing the true (cm) distance travelled by $1/30^{th}$ s (0.0333 s) using the following equation:

$$\frac{\text{Distance (cm) traveled during a single video frame}}{{}^{1}/_{30\text{th}}\ (\text{s})}$$

= instantaneous velocity during a single video frame (cm s^{-1})

ii. The average velocity during the open-field trial was then determined by calculating the mean of the instantaneous velocities for all frames during which the fish was not immobile (i.e. immobile score of 0), using the following Excel function:

=AVERAGEIF("entire immobility column",0,"entire velocity column")

d. Number of bouts of activity: A single bout of activity was defined as one or more consecutive frames during which a fish was active. To quantify the number of these occurrences, a novel Excel Macros script was written by adapting the existing "COUNTIFS" function in Excel. See Appendix E for the Excel macros script. The number of bouts of activity during a whole video trial was determined by counting the instances that the immobility score was "0" (i.e. fish was active) for at least 1 frame, using the following Excel function:

=COUNTINSTANCESGREATERTHAN("entire immobility column",0,1)

Appendix G:

Statistical results for analysis of the length and mass of offspring of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*). Significant effects of maternal treatment are indicated by bold font.

Morphological trait	Corresponding figure	P-value per life-stage	Mean ± SEM		Unit of measurement	P-value
			Control	Cortisol-treated		
Length	Fig. 2.3.a	Embryo	1.51 ± 0.03	1.49 ± 0.07	Mm	0.828 *
		ESF-1	4.18 ± 0.08	4.23 ± 0.17		0.860 *
		ESF-2	5.43 ± 0.30	5.17 ± 0.22		0.487 *
		FSF-1	5.74 ± 0.12	5.97 ± 0.16		0.286 *
		FSF-2	6.37 ± 0.16	6.56 ± 0.25		0.549 *
	Fig. 2.3.b	YOY	5.02 ± 0.32	4.51 ± 0.17	Cm	0.160 *
Mass	Fig. 2.3.c	Embryo	2.13 ± 0.36	2.28 ± 0.27	Mg	0.748 *
		ESF-1	**2.12 ± 0.14**	**2.82 ± 0.18**		**0.011** *
		ESF-2	3.20 ± 0.66	3.77 ± 1.23		0.693 *
		FSF-1	7.80 ± 1.67	5.57 ± 0.39		0.223 *
		FSF-2	6.29 ± 1.29	6.26 ± 1.33		0.387 *
	Fig. 2.3.d	YOY	2.16 ± 0.37	1.24 ± 0.13	G	0.548 †

Embryo, 0 days-post-fertilization (DPF); *ESF*, egg-sac fry, 2-4 DPF; *FSF*, free-swimming fry, 6-8 DPF; *YOY*, young-of-the-year, 35-50 DPF

* Student's *t*-test
† ANOVA including pond as random effect
N = 5-10 nests & 30 YOY

The significant effect of maternal treatment on ESF-1 mass is indicated by bold font.

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