

THE NEUROENDOCRINE AND MOLECULAR MECHANISMS UNDERLYING
STRESS-INDUCED MATERNAL EFFECTS IN THE THREESPINE STICKLEBACK

BY

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DISSERTATION

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ABSTRACT

Non-genetic maternal effects occur when offspring phenotype is influenced non-genetically by a mother's phenotype. Exposure of mothers to external stressors, for example, can influence the expression of a wide array of offspring traits essential for survival, including growth, morphology, and behavior. In fact the exposure of mothers to an ecologically relevant stressor such as a non-lethal predator attack can alter the life-long offspring physiological and behavioral response to the same stressor. The transfer of information about the environment from a mother to her offspring, though, likely depends on the nature of her physical and behavioral contact with them, which in turn depends on the life history of the species. Mothers of many mammalian species, for example, care for offspring postnatally in addition to providing pre- and postnatal resources. The fact that stress-induced maternal effects occur in non-mammalian vertebrates, including many oviparous species that do not exhibit maternal care, indicates that the transgenerational transfer of information can occur entirely through the egg. Little is known, however, about the mechanisms by which mothers transmit information about the environment through the egg or how this information, once received by offspring, influences their phenotype non-genetically.

For my PhD research I studied maternal effects in threespine sticklebacks (*Gasterosteus aculeatus*), with the goal to describe a maternal effect on the physiological stress response and the underlying molecular mechanisms by which the non-genetic maternal effects on physiology and behavior occur in sticklebacks. Sticklebacks are an oviparous fish that do not exhibit maternal care. Further, they are a model species in the study of behavior, easily acclimate and reproduce in a lab setting, and now have a fully sequenced genome, making the species an ideal candidate for the study maternal effects using techniques in genetics, physiology, and behavior. Previous studies in sticklebacks have shown that maternal exposure to predation risk increases the amount of cortisol, a stress hormone, in eggs and affects offspring antipredator behavior, learning, and survival in the presence of live predation.

The physiological stress response, coordinated by the hypothalamus-pituitary-interrenal (HPI) axis, is the biological link between the recognition of external stressors

by the brain and the broad homeostatic changes that occur in the body in response to those stressors. The diversity of offspring behavioral responses to maternal stress in sticklebacks therefore suggests organizational effects on the HPI axis, though to date this has not been tested. Therefore the first chapter of my thesis (Chapter 2) tested the hypothesis that maternal exposure to predation risk has long-lasting effects on the stress response of offspring in sticklebacks. To accomplish this I exposed adult offspring of predator-exposed mothers and control mothers to a simulated predator attack and measured circulating cortisol at multiple time points. Similarly I compared the cortisol responses between sticklebacks in two different social environments (alone or in a group), and between the sexes. The cortisol response curve differed between offspring of control and predator-exposed mothers, between females and males, and between fish alone and fish in a group. There was no interaction between the social environment and maternal treatments, sex and maternal treatment, or social environment and sex. Altogether these results show that, by some mechanism, maternal exposure to a predator influences development of the stickleback HPI axis.

It is likely that activation of the HPI axis in female sticklebacks exposed to stressful events during oogenesis exposes her eggs to elevated cortisol and that her eggs take up some of that extra cortisol. The observation that stickleback mothers exposed to a predator lay eggs with higher cortisol levels is consistent with this idea, though whether stickleback eggs take up cortisol based on the concentration of their surroundings has not been tested. Therefore in the second chapter of my thesis (Chapter 3) I tested the hypothesis that exogenous hormones passively diffuse into eggs. I reviewed the literature on experimental immersion of fish eggs into steroids and found that exogenous glucocorticoid and sex steroids diffuse into both fertilized and unfertilized fish eggs. I also immersed unfertilized stickleback eggs in cortisol and testosterone and measured their levels after exposure. The data confirm that stickleback eggs also exhibit a pattern of diffusion depending on their surrounding concentration, and confirm that the transfer of maternal hormones via diffusion is a potential mechanism underlying the maternal effect in sticklebacks.

Finally, the fact that stickleback mothers' experiences with a predator affect the HPI responsiveness of their adult offspring despite the absence of maternal care in the

species indicates that, in early stickleback embryos, the developing HPI axis and brain remain sensitive to maternal stress. In the final chapter of my thesis (Chapter 4) I uncover some of the molecular mechanisms by which maternal predator exposure influences offspring development. A comparison of genome-wide transcription patterns between embryos from predator-exposed and control mothers demonstrates that early stickleback embryos have a significant transcriptomic response to maternal stress. Developmental pathways involved in metabolism, growth, epigenetics, neural development, sexual maturation, and a role for microRNAs were all identified.

In conclusion, the data I obtained in my PhD research identifies a non-genetic maternal effect on the physiological stress response of offspring, a probable neuroendocrine mechanism whereby stickleback mothers transmit information about the presence of predators in the environment to their offspring, and a set of molecular mechanisms whereby this information, once received by developing offspring, influences their phenotype non-genetically.

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TABLE OF CONTENTS

CHAPTER 1: GENERAL INTRODUCTION	1
CHAPTER 2: A TEST OF MATERNAL PROGRAMMING OF OFFSPRING STRESS RESPONSE TO PREDATION RISK IN THREE SPINE STICKLEBACKS	6
CHAPTER 3: THE USE OF IMMERSION TO MANIPULATE STEROID LEVELS IN FISH EGGS.....	19
CHAPTER 4: PROGRAMMING OF THE EARLY EMBRYONIC TRANSCRIPTOME BY MATERNAL STRESS IN THREE SPINE STICKLEBACKS	27
CONCLUSIONS AND FUTURE DIRECTIONS.....	47
FIGURES AND TABLES	52
BIBLIOGRAPHY	70

CHAPTER 1: GENERAL INTRODUCTION

Non-genetic maternal effects, or the influence of a mother's phenotype on her offsprings' phenotype are widespread across taxa (Agrawal 2002; Fox et al 1999; Kaplan 1992; Sheriff et al 2009). Exposure of mothers to external stressors, for example, can influence the expression of a wide array of offspring traits essential for survival including growth (McCormick 2006; McCormick 2009; Sheriff *et al.* 2009), morphology (Saino *et al.* 2005; Gagliano & McCormick 2009; Sheriff *et al.* 2009), and behavior (Clarke & Schneider 1993; Nishio et al. 2001; Shine & Downes 1999). In fact the exposure of mothers to an ecologically relevant stressor such as a non-lethal predator attack can alter the life-long offspring physiological and behavioral response to the same stressor (Storm and Lima 2010; Green *et al.* 2011; Clarke et al 1994). Predators are one of the most important naturally-occurring stressors for animals in natural populations (Canoine et al 2002; Blanchard et al 1998), and predator-induced maternal effects have been well documented (Shine and Downes 1999; Storm and Lima 2010; Agrawal et al. 1999; Gilbert 1966; Weisser et al. 1999). If the lasting influence of maternal exposure to a predator allows offspring to respond more favorably to the presence of predators in their environment, then there is potential for such maternal effects to be adaptive (Fox et al 1999; Storm and Lima 2010), though there are clear examples where stress-induced maternal effects can be maladaptive for offspring (Kaplan 1992; human disease: Barker 1995; Sie et al 2011).

The transfer of information about a mother's environment, including information about predators, likely depends on the nature of a her physical and behavioral contact her offspring, which in turn depends on the life history of her species. Mothers of many mammalian species, for example, care for offspring postnatally in addition to providing pre- and postnatal resources (Champagne et al 2003). The fact that stress-induced maternal effects occur in non-mammalian vertebrates, including many oviparous species that do not exhibit maternal care (Shine and Downes 1999; Groothuis and Schwabl 2008; Giesing et al 2011), indicates that the transgenerational transfer of information can occur entirely through the egg. Little is known, however, about the mechanisms by which

mothers transmit information about the environment through the egg or how this information, once received by offspring, influences their phenotype non-genetically.

A potential mechanism of information transfer from mothers to offspring is the observation that offspring appear to be sensitive to the effects of maternal hormones. For example, prenatal exposure to maternally-derived stress hormones is known to have lifelong consequences if it occurs during sensitive periods in development when the HPA (hypothalamic-pituitary-adrenal) axis, or in fishes, HPI (hypothalamic-pituitary-interrenal) axis is developing (reviewed in: Weinstock 2008; Matthew & Phillips 2010; Jobe et al 1998; Hu et al 2008). If prenatal exposure to stress hormones has organizational effects, that is, if they influence differentiation of the developing brain (Phoenix et al 1959; Arnold and Breedlove 1985; Arnold 2009), then early exposure to maternally-derived stress hormones might impact an individual's stress response for life.

To investigate the role that exposure to maternal hormones plays on the development of offspring traits many studies have focused on the sensitivity of embryonic gene expression to exogenously applied hormones (e.g. cortisol, Li et al 2010; estradiol, Chandrasekar et al 2010) or hormone-disrupting toxins (e.g. bisphenol A, Aluru et al 2010). For example exposure of early rainbow trout embryos to exogenous cortisol has been shown to elevate the expression levels of thyroid hormone receptor beta ($Tr\beta$) in embryos, and to accelerate their growth (Li et al 2010). Conversely, exposure of rainbow trout embryos to bisphenol A leads to decreased expression of insulin-like growth factor genes (IGF-1 and IGF-2) and suppression of embryonic growth (Aluru et al 2010). The effect of hormonally-mediated maternal effects on offspring gene expression can also be tissue specific. Several studies, for example, have found a significant effect of maternal treatment on brain-specific gene expression in adult rodent and teleost fish offspring (serotonin, Mueller and Bale 2008; ACTH, Leatherland et al 2010; glucocorticoid receptor, Szyf et al 2005; estrogen receptor, Leatherland et al 2010). Measuring changes in the expression of genes in embryos that result from exposure to maternal stress or exogenous hormones is a promising method to understand the earliest manifestations of offspring phenotypic change, however much of the work thus far has focused on only a small set of genes. Genome-wide transcriptome analysis is a promising approach to

address this question: it is an open-ended and unbiased approach and can identify molecular mechanisms that respond to maternal stress that are currently unknown.

Maternal effects in threespine sticklebacks

For my PhD research I have studied maternal effects in threespine sticklebacks (*Gasterosteus aculeatus*), an oviparous fish that does not exhibit maternal care. Sticklebacks are well-suited for investigating hormonally-mediated maternal effects on the stress response. Levels of cortisol in sticklebacks increases following exposure to a model predator (Bell et al. 2007) and some of this cortisol appears to be taken up by eggs, affecting offspring behavior (Giesing et al. 2011; McGhee et al 2012) learning (Roche et al 2012), and survival (McGhee et al 2012) later in life. Stickleback mothers are not thought to have contact with offspring after laying eggs, so the maternal effect on behavior in this species is not mediated by maternal care. Decades of behavioral research on sticklebacks including, more recently, personality research (Bell and Sih, 2007) offer evolutionary context to studies on the molecular mechanisms underlying maternal effects. There is now a stickleback reference genome with gene-level annotation (Jones et al. 2012) that allows measurement of genome-wide transcripts. This system therefore provides the opportunity to link maternal experience with a predator to specific molecular pathways underlying the expression of maternal effects.

The physiological stress response in sticklebacks, coordinated by the hypothalamus-pituitary-interrenal (HPI) axis, is the biological link between the recognition of external stressors by the brain and the broad homeostatic changes that occur in the body in response to those stressors (Alsop and Vijayan 2009). The presence of diverse effects of maternal stress on stickleback behavior therefore suggests that offspring of stressed mothers have altered HPI axis function, though to date this has not been tested. Also, it is likely that activation of the HPI axis in female sticklebacks exposed to stressful events during oogenesis exposes her eggs to elevated cortisol and that her eggs take up some of that extra cortisol. The observation that stickleback mothers exposed to a predator lay eggs with higher cortisol levels (Giesing et al 2011) seems to support this idea, though whether stickleback eggs take up cortisol based on the concentration of their surroundings has also not been tested. Finally, if stickleback

mothers' experiences with a predator affect the HPI responsiveness of their adult offspring despite the fact that sticklebacks do not exhibit maternal care, it would indicate a sensitivity of the developing HPI axis and brain of early embryos to maternal stress.

In Chapter 2 I test the hypothesis that maternal exposure to predators has long-lasting effects on the HPI response to predators in stickleback offspring. To accomplish this I compare the cortisol response of adult offspring of predator-exposed mothers and control mothers to a simulated predator attack. By measuring circulating concentrations of cortisol before (baseline), 15 minutes after, and 60 minutes after exposure to a simulated predation risk I was able to compare the shape of the stress response curve between maternal treatments, between two different social environments (alone or in a group), and between the sexes. Relative to baseline, offspring plasma cortisol was highest 15 minutes after exposure to simulated predation risk and decreased after 60 minutes, as expected in a vertebrate stress response. Offspring of predator-exposed mothers differed in the shape of the cortisol response to simulated predation risk compared to offspring of control mothers. In general, females had higher cortisol than males, and fish in a group had lower cortisol than fish that were by themselves. The buffering effect of the social environment did not differ between maternal treatments or between males and females. Altogether these results show that, by some mechanism, maternal exposure to a predator influences development of the stickleback offspring HPI axis and its reactivity to predator exposure, though not to the magnitude that sex and the presence of a social group affect plasma cortisol.

In Chapter 3 I test the hypothesis that stickleback eggs take up cortisol depending on its concentration in their surroundings. There are a number of studies in a diverse array of fish species that have measured steroid uptake by eggs, but these data have not been summarized across species or across steroids. Therefore I reviewed the literature on exogenous manipulations of steroid levels in fish eggs to discern general patterns. The literature review suggests that exogenous steroids diffuse into both fertilized and unfertilized fish eggs. Finally, I applied exogenous cortisol and testosterone to unfertilized stickleback eggs and measured their levels before and after exposure to discern rate of uptake. The data confirm that stickleback eggs exhibit the same pattern as the fish species from the literature, and suggest that maternal steroids likely enter

stickleback eggs via diffusion. These results provide a useful resource for the investigation of hormonal mediation as a mechanism underlying maternal effects by allowing researchers to predict the concentrations of steroid needed to raise levels in eggs to a specified amount to simulate maternal stress in vitro.

The link between elevated cortisol in eggs of stickleback mothers exposed to a predator and offspring behavior and learning suggests that predator-exposed mothers produce eggs with more cortisol and that cortisol affects the development of the brain or HPI axis in stickleback embryos. In Chapter 4 I test this hypothesis by comparing genome-wide transcription patterns of embryos of predator-exposed mothers to embryos of control mothers. The results demonstrate that early stickleback embryos have a significant transcriptomic response to maternal stress, including differential regulation of many genes and pathways involved in metabolism, growth, epigenetics, neural development, and sexual maturation. The absence of expression in early stickleback embryos of several genes responsible for HPI axis function suggests that the offspring stress response is not yet active, as recent data on the consistently low, unchanging level of cortisol during ontogeny in sticklebacks seems to confirm (Paitz et al, in prep). The highly sensitive RNA-seq approach used in this experiment found several genes that have previously been overlooked or have received little notice due to their low levels of expression, but nevertheless exhibit strong differences between maternal treatments, including several microRNAs. These results highlight specific structures and functions in the developmental pathway that are sensitive to maternal exposure to predation risk, and the genes underlying them. Understanding how embryos react to the changing maternal environment shapes the way we understand societal problems such as the prevalence of drug and alcohol dependency in human populations (reviewed in Diaz-Anzaldúa et al 2011) and the toxicity of substances we release into the environment (reviewed in Fent and Sumpter 2011; Yang et al 2007).

CHAPTER 2: A TEST OF MATERNAL PROGRAMMING OF OFFSPRING STRESS RESPONSE TO PREDATION RISK IN THREESPINE STICKLEBACKS¹

Abstract

Non-genetic maternal effects are widespread across taxa and challenge our traditional understanding of inheritance. Maternal experience with predators, for example, can have lifelong consequences for offspring traits, including fitness. Previous work in threespine sticklebacks showed that females exposed to simulated predation risk produced eggs with higher cortisol content and offspring with altered anti-predator behavior. However, it is unknown whether this maternal effect is mediated via the offspring glucocorticoid stress response and if it is retained over the entire lifetime of offspring. Therefore, we tested the hypothesis that maternal exposure to simulated predation risk has long-lasting effects on the cortisol response to simulated predation risk in stickleback offspring. We measured circulating concentrations of cortisol before (baseline), 15 minutes after, and 60 minutes after exposure to a simulated predation risk. We compared adult offspring of predator-exposed mothers and control mothers in two different social environments (alone or in a group). Relative to baseline, offspring plasma cortisol was highest 15 minutes after exposure to simulated predation risk and decreased after 60 minutes. Offspring of predator-exposed mothers differed in the cortisol response to simulated predation risk compared to offspring of control mothers. In general, females had higher cortisol than males, and fish in a group had lower cortisol than fish that were by themselves. The buffering effect of the social environment did not differ between maternal treatments or between males and females. Altogether the results show that while a mother's experience with simulated predation risk might affect the physiological response of her adult offspring to a predator, sex and social isolation have much larger effects on the stress response to predation risk in sticklebacks.

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Introduction

Non-genetic maternal effects occur when conditions experienced by mothers influence the phenotype of their offspring (Mousseau and Fox 1998). Maternal effects occur in diverse taxa (plants, Agrawal 2002; insects, Fox et al 1999; amphibians, Kaplan 1992; mammals, Sheriff et al 2009) and their effect on offspring fitness can be maladaptive (Kaplan 1992; Barker 1995; Sie et al 2011) or adaptive (Fox et al 1999; Storm and Lima 2010). There is growing evidence that some maternal effects are mediated by maternal steroid hormones (sex steroids, Groothuis et al 2005; glucocorticoids, Uller et al 2009). For example high levels of circulating glucocorticoids in stressed mothers are transferred to developing eggs in birds, affecting offspring phenotype (Groothuis and Schwabl 2008).

Studies in diverse taxa have shown that maternal stress can have long-lasting effects on offspring traits including survival (Sheriff et al 2009; Gagliano and McCormick 2009; Saino et al 2005; McGhee et al 2012), growth (Sheriff et al 2009; McCormick 2006; McCormick 2009), morphology (Sheriff et al 2009; Gagliano and McCormick 2009; Saino et al 2005), learning (Roche et al 2012), and behavior (Roche et al 2012; Clarke and Schneider 1993; Nishio et al 2001; Shine and Downes 1999). In addition, some studies have shown long-lasting effects of maternal stress on the offspring glucocorticoid response to stressors (Barbazanges et al 1996; Hu et al 2008), suggesting that maternal steroids have organizational effects (Phoenix et al 1959) on the development of the offspring hypothalamus-pituitary-adrenal (HPA) or, in fishes, hypothalamus-pituitary-interrenal (HPI) axis that persist throughout an offspring's lifetime. Therefore it is possible that mothers might 'programme' their offspring for the types of environments they are likely to experience later in life (Darnaudéry and Maccari 2008).

Predators are one of the most important naturally-occurring stressors for animals in natural populations (Blanchard et al 1998; Canoine et al 2002). Predator-induced maternal effects have been documented in diverse taxa, with effects on multiple offspring traits (Sheriff et al 2009; Storm and Lima 2010; Shine and Downes 1999; Agrawal et al 1999; Gilbert 1966; Weisser et al 1999). A previous study on threespine stickleback fish found that predator-exposed mothers produced offspring that exhibited higher levels of

shoaling behavior than offspring of control mothers (Giesing et al 2011). Shoaling involves swimming in close proximity to conspecifics, resulting in the formation of groups (Masuda et al 2003), and is an effective antipredator defense in small fishes (Magurran 1990). Female sticklebacks exposed to simulated predator attacks also produced eggs with higher concentrations of cortisol (Giesing et al 2011). Predator exposure triggers the release of cortisol in sticklebacks (Bell et al 2007), therefore a plausible explanation for the maternal effect observed by Giesing et al. (2011) is that maternally-derived cortisol diffused into eggs and induced an organizational effect on the HPI axis during offspring development. If this is the case, then offspring of predator-exposed mothers might have an altered cortisol response to a stressor compared to offspring of control mothers.

Other studies have shown a buffering effect of the social environment on the cortisol response to stressors (cows, Boissy and Neindre 1997; pigs, Kanitz et al 2009; guinea pigs, Machatschke et al 2004; gorillas, Stoinski et al 2002). Therefore we hypothesized that a maternal effect on the stickleback cortisol response might differ according to the social environment. For example, offspring of predator-exposed stickleback mothers might shoal together in order to cope with simulated predation risk, and might perceive simulated predation risk as more threatening if they do not have the opportunity to shoal.

In this study we tested the maternal programming hypothesis in sticklebacks. Specifically, we examined whether maternal experience with a simulated predation risk by Northern pike (*Esox lucius*), a natural predator of sticklebacks, influences offspring cortisol response to simulated predation risk by pike. We measured circulating plasma cortisol of adult offspring of predator-exposed and unexposed mothers before (baseline), 15 minutes after and 60 minutes after exposure to simulated predation risk by pike. We predicted that offspring of predator-exposed mothers have higher baseline and predator-induced cortisol than offspring of control mothers. To test the hypothesis that the presence of a social group during exposure to simulated predation risk buffers the glucocorticoid response of offspring, we compared adult offspring of predator-exposed and unexposed mothers that were either alone or in a group at the time of exposure to simulated predation risk. Finally, hormonally-mediated maternal effects are often sex-

specific (Blanchard et al 1998; Canoine et al 2002; McMullen and Langley-Evans 2005; Zohar and Weinstock 2011). For example, pregnant mice exposed to stressors during the first week of gestation gave birth to male offspring that as adults exhibited a larger glucocorticoid response than male offspring of control mothers, an effect not seen in female offspring (Mueller and Bale 2008). Therefore we also investigated the influence of sex on the cortisol response to predation risk.

Materials And Methods

Maternal predator exposure. Adult threespine sticklebacks were collected from Putah Creek, CA in May 2010 and were acclimated to the lab for at least one month before experiments began. Throughout, all fish were maintained in a flow-through system with UV, charcoal, particulate and biological filters that remove olfactory cues and a photoperiod that mimics seasonal changes. Fish were fed *ad libitum* once daily frozen bloodworms, brine shrimp, mysis shrimp, and cyclopeez, and uneaten food removed at the end of the day.

Females were exposed to simulated predation risk by exposing them to a model pike as in McGhee et al. (2012). Briefly, they were housed in 38 L tanks (53L x 33W x 24H cm) with artificial plant refuges, gravel bottom and opaque external shading. Females were randomly assigned to one of six 38L tanks ('treatment tanks') at a density of five females per tank and either exposed to simulated predation risk ('predator-exposed' mothers) or left undisturbed ('control' mothers) until they became gravid. Predator-exposed females were chased for 30 seconds/day at a randomly-chosen time with a painted clay model of Northern pike (23cm standard length) to simulate exposure to predation risk (Giesing et al 2011). Predator-exposed mothers were chased once daily for 56 ± 21 days (mean $\pm$ standard deviation, range 22 to 85 days) before being stripped of eggs. Consistent with a previous study (Giesing et al 2011), there was no effect of the number of days in the maternal treatment on offspring cortisol (results not shown). Live Northern pike have been shown to elicit a cortisol response in sticklebacks (Bell et al 2007) and simulated attack by a model pike has been shown to elicit antipredator behaviors as compared to a non-threatening stimulus (Grobis et al 2013). Pike do not inhabit the section of Putah Creek where sticklebacks were caught; therefore we know

that mothers did not have experience with pike before the experiment. When females became gravid, they were stripped of eggs by hand and were replaced by a marked female in order to maintain the same density.

Testes were dissected from wild-caught males from the same population, macerated to release sperm, and the sperm was used to fertilize eggs. Sperm was used from nine different males: sperm from three males fertilized eggs from control mothers only (n=5 clutches), sperm from three other males fertilized eggs from predator-exposed mothers only (n=5 clutches), and sperm from three additional males fertilized eggs from both control and predator-exposed mothers (n=10 clutches). Altogether, clutches were collected from n=10 predator-exposed mothers and n=10 control mothers. After contributing one clutch of eggs, females were no longer used in the experiment.

Rearing offspring. Offspring rearing for this experiment was carried out as described in McGhee et al (2012). Briefly, fertilized eggs were artificially incubated in plastic cups with air bubblers. Juveniles were transferred to 38L tanks ('tank of origin', 53L x 33W x 24H cm) surrounded by opaque shading and artificial plants for refuge in either single family groups (n=11 families) or mixed family groups (n=9 families) within each maternal treatment. Fry were fed newly-hatched brine shrimp nauplii. Offspring were reared in their tank of origin until they entered the simulated predation risk experiment (see below) in August 2011, when they were approximately one year old (standard length 3.83 ± 0.39 cm mean $\pm$ standard deviation). On average, the number of offspring surviving to adulthood per mother did not differ between maternal treatments: control mothers had 60.5 ± 15.9 surviving offspring (mean $\pm$ standard error) per mother and predator-exposed mothers had 61.2 ± 9.9 surviving offspring per mother.

The number of offspring used in the experiment from each family housed in single family tanks did not differ between maternal treatments. 14.33 ± 5.37 offspring (mean $\pm$ standard error) per mother were used from control mothers and 14.83 ± 3.54 offspring per mother used from predator-exposed mothers. Mixed and single family groups of offspring were equally represented across all treatments. Since individuals in mixed family tanks were not identified by parentage, it is possible that some families were overrepresented in the mixed family tanks.

Simulated predation risk experiment. Twelve 38 L observation tanks (53L x 33W x 24H cm) with artificial plant refugia and surrounded on the sides with opaque material to minimize the effect of external visual stimuli were used for the offspring simulated predation risk experiment. Tanks were assigned to one of three different ‘time’ treatments for sampling: baseline, 15 minutes and 60 minutes, with n=4 replicate tanks per treatment. Focal fish in the ‘baseline’ tanks were not exposed to simulated predation risk, focal fish in the ‘15 minutes’ tanks were exposed to simulated predation risk and sacrificed for cortisol 15 minutes later and focal fish in the ‘60 minutes’ tanks were exposed to simulated predation risk and sacrificed for cortisol 60 minutes later (described further, below).

Half of the 12 tanks were randomly assigned to one of two different social environment treatments (alone vs. group) with the constraint that the two social environments were equally distributed between the different ‘times’. The ‘group’ tanks contained four ‘background’ individuals from a pool of approximately 30 fish that were maintained in two 38 L holding tanks when not being used in the experiment. The background fish were spine-clipped one week prior to the experiment in order to distinguish them from the focal fish, which were unmarked. The ‘alone’ tanks did not contain background individuals.

Twelve focal sticklebacks were randomly assigned to the 12 observation tanks with one focal fish per observation tank, with the constraint that maternal treatments were equally represented across social environments and time and that no more than one individual was drawn from each tank of origin per day. In order to avoid pseudoreplication, there was only one focal fish per observation tank in the ‘group’ treatment. Focal fish were acclimated to the observation tanks for two hours, an amount of time previously found to be sufficient for stickleback whole body cortisol to reach and maintain a stable 24-hour level after handling and transfer (Pottinger et al 2002). Individuals in the ‘baseline’ tanks were sacrificed immediately after the two-hour acclimation period. Fish in the ‘15 minutes’ and ‘60 minutes’ tanks were exposed to simulated predation risk. A deceased and frozen Northern pike (Spirit Lake Fish Hatchery, Spirit Lake, IA; SL=18.5cm, mass = 68.8g) was thawed, suspended from a dowel with fishing line, and used to simulate the predation risk, which consisted of

repeated rounds of orientation, slow approach, and lunging at the stickleback for 60 seconds. Although different procedures were used to apply simulated predation risk to mothers (model pike) and their offspring (frozen pike), we assume that the model pike and the frozen pike were both perceived as a threatening pike predator by the sticklebacks (Grobis et al 2013). The pike was frozen and thawed between successive days of the experiment.

Either 15 or 60 minutes after applying simulated predation risk, the opaque shading was removed from the observation tank in order to distinguish focal from background individuals, the focal fish was netted from the observation tank and immediately sacrificed with a fatal concentration ($>0.2\text{mM}$) of MS-222 anesthetic. Time to opercular arrest was <30 seconds and the time from shade removal to opercular arrest was <60 seconds. After opercular arrest, the fish was blotted with paper towel, standard length was measured, and the caudal peduncle was severed posterior to the cloaca. Blood was drawn by capillary action from the caudal vein into a 75mm heparinized microhematocrit tube (Statspin, Westwood, MA). Tubes were centrifuged on a microhematocrit rotor (Statspin, Westwood, MA) to pellet circulating cells, and plasma supernatant aspirated to a microfuge tube and stored at -20°C . A small sample of muscle tissue was taken from the tail for determination of genetic sex using a sex-specific genetic marker (Peichel et al 2001).

This procedure (random assignment of observation tanks, addition of $n=12$ focal animals to the observation tanks) was repeated 15 times between 2-17 August, 2011. On some days ($n=6$ days), the procedure was repeated twice, with at least two hours between procedures. In two hours, the water in the observation tanks was replaced at least four times; therefore there was no carryover of olfactory cues between procedures (82.2 ± 6.0 L/hour mean $\pm$ SEM flow rate). Altogether we sampled $n=15$ focal individuals per maternal treatment * time * social environment combination.

Individual offspring that entered the simulated predation risk experiment were not replaced, so the density of offspring in tanks of origin decreased over time as the experiment progressed. The density of offspring in the tanks of origin at the start of the offspring experiment was 10.0 ± 0.94 fish (mean $\pm$ standard error) and at the end of the experiment was 3.36 ± 0.65 fish. Feeding rates were adjusted according to density.

Mortality of adult offspring during the course of the offspring simulated predation risk experiment was very low (number of deaths: offspring of control mothers = 0.25 ± 0.16 adults per mother (mean $\pm$ standard error); there were no deaths of adult offspring of predator-exposed mothers).

Cortisol assay. Plasma samples were thawed and their cortisol concentration measured in duplicate by competitive enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Enzo Life Sciences, Plymouth Meeting, PA). The antibody used in this assay is reported by the manufacturer to have a sensitivity of 56.72 pg/ml and a 27.7% cross reactivity with corticosterone, 4.0% with 11-deoxycortisol, 3.6% with progesterone, and 0.1% or less with testosterone, androstenedione, cortisone, and estradiol. Samples of adequate volume ($\sim 4\mu\text{l}$) were treated with 1:10 dilution of steroid-displacement reagent to free cortisol from its binding proteins, diluted to 1:50 sample:assay buffer ratio, and divided in half to be measured as duplicates. To extend the manufacturer's recommended standard curve for the ELISA an 8th standard of cortisol (78 pg/mL) was included on each plate. Following the manufacturer's protocol, the plates were prepared and absorbance read at 405nm for each sample in duplicate in a 96-well ELISA plate (Enzo Life Sciences, Plymouth Meeting, PA) on a FilterMax F3 microplate reader (Molecular Devices, Sunnyvale, CA) and absorbance data averaged across duplicate samples with Multi-Mode Analysis software (Molecular Devices version 3.4.0.25).

Blank-corrected, total-activity-corrected optical densities were converted to 'percent bound' for each sample by applying an equation derived from each plate's standard curve. Both linear and four-factor polynomial standard curves were produced for each ELISA plate and tested for accuracy in calculating sample cortisol concentration from 'percent bound' value of the known standards. The polynomial method (which is recommended by the manufacturer) fit the expected concentration of the standards more closely than the linear method, and so was employed for the final analysis. Values were multiplied by their dilution factor to obtain plasma cortisol concentration in ng/mL of the original, undiluted plasma sample.

Plasma samples across all levels of maternal treatment (predator-exposed and control), social environment (alone and group), and time (0, 15, and 60) were represented

on each of the six plates. The inter-assay coefficient of variation (CV) between all ELISA plates (n=6 plates) was 2.40%; intra-assay CVs averaged $5.02 \pm 2.37\%$ (mean $\pm$ standard deviation). Six individuals either gave very little blood or the plasma was lost from the capillary tube during isolation, therefore the final sample size was 174 focal individuals.

Statistical analysis. All analyses were performed using SAS version 9.3. The cortisol data were ln-transformed to meet the assumptions of parametric tests. Cortisol data were analyzed with a general linear model using the “proc GLM” command in SAS. We started with a full model that included sex, social environment, time, and maternal treatment as fixed effects, date as a covariate and all the two- and three-way interactions. ‘Date’ was the day in August 2011 when a sample was collected. Terms with p-value > 0.05 were step-wise removed from the model, unless their interaction with another variable was statistically significant ($P < 0.05$). We plotted $\ln\text{cort}$ against date for each level of time, and also for each level of maternal treatment*social environment*time, and we found no significant interactions between the covariate and these variables (data not shown).

Although the analyses were carried out on ln-transformed data, for ease of comparison with other studies we show the raw (untransformed) data in figures. Post-hoc tests were performed on a pairwise basis on all terms deemed significant by the statistical model. Least squares estimates of the mean of each term were obtained using the LSMEANS statement in SAS, and were compared between levels within each term.

Results

Relative to baseline, levels of plasma cortisol increased 15 minutes after exposure to simulated predation risk and dropped by 60 minutes (Table 2.1, main effect of time). We did not detect a main effect of maternal treatment on concentrations of cortisol. However, maternal exposure to simulated predation risk influenced the time course of the cortisol response of offspring (Table 2.1, maternal treatment * time interaction). That is, offspring of predator-exposed mothers mounted a different cortisol response to simulated predation risk than the offspring of control mothers (Figure 2.1). Post-hoc tests

comparing least squares means between levels of maternal treatment*time were non-significant (p-values > 0.05).

By comparison there was a relatively large effect of sex on plasma cortisol. Females had higher plasma cortisol than males (29.4 ± 4.3 ng/ml vs. 14.3 ± 1.7 ng/ml, Table 2.1, main effect of sex). We did not detect an interaction between sex and social environment; females had higher plasma cortisol in both the 'alone' and 'group' treatments (Figure 2.2).

There was a strong buffering effect of the presence of a social group on the cortisol response to simulated predation risk; fish that were in a group had lower levels of plasma cortisol than fish that were by themselves (Figure 2.3, Table 2.1 main effect of social environment). However there was no evidence that the social environment had more of a buffering effect on the response to a stressor of offspring of predator-exposed mothers: there was not a statistically significant interaction between the social environment and maternal treatment (Table 2.1).

There was an effect of date on plasma cortisol (Table 2.1): fish that were sampled later in the experiment had lower concentrations of cortisol than fish that were sampled at an earlier date. There were no significant interactions between date and maternal treatment, social environment, sex, or time (all p-values > 0.05).

Mean effect size sizes for several studies that measured the glucocorticoid response to stressors in offspring exposed to either real or simulated maternal stress and compared to control offspring was 0.895 (range 0.317 to 2.189) (Marasco et al 2012; Haussmann et al 2012; Park et al 2003; Hayward et al 2006; Love and Williams 2008). Power analysis using our sample size confirmed that our study had high power (mean=0.866) to detect the effect sizes of published studies.

The range of plasma cortisol levels observed in this study (22.00 ± 2.23 ng/ml SEM) are similar to whole body cortisol measured in sticklebacks (Bell et al 2007; Pottinger et al 2002), and were similar to the natural range between baseline and predator-induced levels observed in the plasma of other teleost species (25-110 ng/ml in rainbow trout, Flores and Shrimpton 2012; 20-110ng/ml in Atlantic cod, Hori et al 2012).

Discussion

These results show that sticklebacks mounted a cortisol response to simulated predation risk: plasma cortisol increased 15 minutes and then dropped 60 minutes after exposure to simulated predation risk. This pattern differs from another study which found that whole body cortisol continued to increase up to 60 minutes post exposure to simulated predation risk (Bell et al 2007), but is consistent with studies in other animals which have found that the plasma cortisol response to an acute stressor declines by 60 minutes after a stressor (Barbazanges et al 1996; Hu et al 2008). The high cortisol levels 60 minutes after exposure to a predator observed in Bell et al. (2007) likely reflects additional intracellular cortisol and cortisol byproducts in whole body extracts. Our results add to the growing literature on the cortisol response to predators, a naturally-occurring, ecologically-relevant stressor.

We tested the hypothesis that maternal experience with a pike predator influences offspring cortisol response to pike in sticklebacks. The hypothesis was partially supported in that there was a significant interaction between maternal treatment and time. The pattern in Figure 2.1 suggests that offspring of predator-exposed mothers mounted a stronger cortisol response to predation risk (compare offspring of control and predator-exposed mothers at baseline versus at 15 minutes). However, post-hoc comparisons of least squares means between levels of maternal treatment and time were not significant.

We detected a significant sex difference in circulating plasma cortisol: females had higher cortisol than males. This result is similar to findings in other fish including sockeye salmon (Kubokawa et al 1999) and the poeciliid *B. episcopi* (Archard et al 2012), but differs from the roach *R. rutilus*, in which males of the species had higher cortisol during spawning (Vainikka et al 2004). Offspring measured in this experiment were reproductive adults, so it is possible that cortisol levels we observed reflect the naturally-occurring sex differences during spawning in sticklebacks. The lack of interaction between sex and maternal treatment in our study suggests that this maternal effect in sticklebacks does not depend on sex (unlike Blanchard et al 1998; Canoine et al 2002; McMullen and Langley-Evans 2005; Zohar and Weinstock 2011).

We also found that the presence of a social group buffered the cortisol response for all offspring, independent of maternal treatment. A likely explanation for lower levels

of cortisol in a group is the dilution effect: the risk of predation is lower in a group and therefore an individual's perceived vulnerability is lower (Magurran 1990). Although the maternal effect on the cortisol response did not depend on the presence of a social group in this experiment, it is possible that in natural populations where individuals are free to choose their own social environments (as opposed to an experimentally-imposed environment), offspring of predator-exposed mothers might be more likely to actively seek out social groups. Indeed, Giesing et al. (2011) found that offspring of predator-exposed stickleback mothers swam closer to their neighbors than offspring of control mothers.

We detected a significant effect of 'date' on plasma cortisol: fish that were sampled later in the experiment had lower cortisol than fish that were sampled earlier in the experiment. There are at least three non-exclusive explanations for this finding. First, a frozen and thawed pike was repeatedly used to chase offspring. It is possible that olfactory cues present in the pike's skin degraded over the course of the experiment such that sticklebacks exposed to the pike towards the end of the experiment experienced fewer olfactory cues than sticklebacks exposed to the pike towards the beginning of the experiment. Second, individual offspring were not replaced in the tanks of origin therefore sticklebacks measured towards the end of the experiment came from tanks at lower density than focal sticklebacks measured towards the beginning of the experiment. It is possible that fish tested earlier in the experiment which had experienced higher density were more stressed. Finally, it is possible that decreasing concentrations of cortisol over time reflect seasonal changes.

Conclusion

We found that sex, exposure to predation risk, the presence or absence of a social group, and a mother's experience with predators all influence the cortisol content of an adult stickleback's plasma. While sex and the social environment have a clear and strong influence, our data show a more subtle contribution of maternal experience with predation risk. However, these results suggest that if a female stickleback encounters a predator during her lifetime, the experience might influence the lifelong responses of her offspring to predators. The mechanism whereby information about a stickleback mother's

experience with predators is encoded and passed to her offspring to alter their cortisol response to stressors remains to be determined, as does its adaptive significance.

CHAPTER 3: THE USE OF IMMERSION TO MANIPULATE STEROID LEVELS IN FISH EGGS

Abstract

Researchers investigating the effect of maternal steroids on offspring often wish to experimentally manipulate steroids in eggs. In fish this is often accomplished by immersing eggs in a medium containing a predetermined concentration of steroid and then measuring steroid uptake. Understanding the amount of steroid taken up by fish eggs is of importance for studies wishing to obtain a specific level of steroid in eggs. There are a number of studies in a diverse array of fish species that have measured steroid uptake by eggs, but these data have not been summarized across species or across steroids. Therefore we review the literature on exogenous manipulations of steroid levels in fish eggs to discern general patterns. The literature review suggests that exogenous steroids diffuse into both fertilized and unfertilized fish eggs, though in general studies have tended to use concentrations of steroids that are far above physiologically relevant levels. Finally, we exogenously applied cortisol and testosterone to unfertilized stickleback eggs and measured their levels after exposure. Our data confirm that stickleback eggs exhibit the same pattern as the fish species from the literature. Altogether our results provide a useful resource for the investigation of hormonal mediation as a mechanism underlying maternal effects by allowing researchers to predict the concentrations of steroid needed to raise levels in eggs to a specified amount.

Introduction

Exogenous steroids can be applied to fish eggs to manipulate steroid levels, creating different steroid environments. This can be a useful technique for studies that wish to simulate increased exposure to maternally derived steroids, or to endocrine-disrupting chemicals (EDCs) that eggs might encounter in the environment (reviewed in Finn 2007). Many studies have measured the level of a steroid in fish eggs both before and after exogenous application of that steroid and concluded that diffusion into eggs has occurred (Table 3.1). However it is unclear whether eggs of different fish species or

different steroids exhibit similar patterns of diffusion. Understanding the relationship between the level of steroid eggs are exposed to and the amount they take up is important for studies wishing to evaluate the transfer of steroids from mothers to developing offspring, for example studies correlating levels of cortisol in the plasma of stressed mothers and their eggs (Stratholt et al 1997).

There is growing evidence in several vertebrate species that the transfer of hormones from mother to offspring can mediate non-genetic maternal effects (Groothuis and Schwabl 2008). In oviparous species such as fish, the transfer of steroid hormones to offspring is thought to occur continuously until the point eggs are laid by some combination of passive diffusion from a mother's circulation and active deposition by ovarian cells to the developing lipophilic yolk (Groothuis and Schwabl 2008; Moore and Johnston 2008). Elevated concentrations of steroids in fish eggs, such as cortisol (Gagliano and McCormick 2009) and testosterone (Burton et al 2011) are known to affect offspring growth and behavior, suggesting that the level of maternal steroids in eggs can have profound influence on embryonic development. For example, the level of maternally-derived cortisol in eggs of tropical damselfish (McCormick 1998) and stickleback fish (Giesing et al 2011) depends on whether a mother was exposed to stress from a predator and is associated with morphological and behavioral differences in offspring.

In this paper we review the literature to determine whether exogenously-applied steroids diffuse into fish eggs. Then, we performed an experiment to determine whether the eggs of an emerging model organism (threespined sticklebacks (*Gasterosteus aculeatus*)) take up the steroids cortisol and testosterone via diffusion. In particular, we tested whether exposure to relatively low levels of exogenous steroids, typical of what eggs would be exposed to *in utero*, is sufficient to manipulate steroid concentrations in eggs. Therefore stickleback eggs were exposed to exogenous cortisol or exogenous testosterone at one of three different concentrations 0 ug/L, 10 ug/L, and 500 ug/L, hereafter referred to as “control”, “low”, and “high”. The low concentration is slightly below the average naturally occurring plasma cortisol levels in female sticklebacks (roughly 15-40 ug/L; Mommer and Bell 2013). The high concentration is typical of studies that have manipulated egg steroid levels via immersion (Table 3.1).

Methods

Literature review. We searched the scientific literature for studies that compared the level of a steroid hormone before and after its exogenous application to fish eggs. First we compiled a list of potential studies using keyword searches (“steroid”, “egg”, “oocyte”, “fish”, “uptake”, “exogenous”) of the electronic database ISI Web of Science (Thomson Reuters, accessed February 2013). We also found articles by searching the list of cited references within each article and the list of articles citing the article. Original articles were included in the analysis if they reported data on steroid concentration in eggs for at least one steroid treatment and one control. For each study, we recorded the species, whether the eggs were fertilized, the steroid applied (cortisol (F), testosterone (T), 17 β -estradiol (E2)), the concentration(s) applied, the duration of application, the sample size, the type of media, and the method of measurement (RIA, ELISA, etc). When steroid concentration data were not presented in the text or tables we estimated it from figures. While several studies have measured the uptake of thyroid hormone triiodothyronine (T3) by fish eggs (Raine et al 2004, Tagawa and Brown 2001) as a non-steroidal hormone the T3 molecule is not expected to exhibit a similar pattern of solubility as steroids. Therefore we restricted our comparison to F, T, and E2.

The majority of studies reported steroid concentrations in pg/egg. We converted the data to ng/g if the egg mass data were also reported or if we could obtain egg mass data from the authors. Studies from which egg mass data could not be obtained (Auperin and Geslin 2008; Stratholt et al 1997) were not included in Figure 3.1 but are in Table 3.1. We refer to the difference between concentrations of steroids measured in control and exposed eggs as “net uptake”.

Because the duration of exposure and concentrations of steroid applied were highly variable, for ease of comparison we converted exposure duration and concentration to hours and ng/L, respectively. To control for the effect of exposure duration we combined exposure duration and concentration into a single term, ng*hr/L, which we refer to as “steroid exposure”. “Net uptake” and “steroid exposure” both range several orders of magnitude, therefore to aid in visualization, we log-transformed both. To discern the general relationship between levels of steroids in eggs before and after exposure we plotted log(steroid exposure) against log(net uptake) (Figure 3.1).

Stickleback egg steroid exposure. Juvenile threespine sticklebacks were collected from Putah Creek, CA in May 2010 and were acclimated to the laboratory at the University of Illinois for at least one year. All fish were maintained in 38 L tanks (53L x 33W x 24H cm) connected to a flow-through system with UV, charcoal, particulate and biological filters that remove olfactory cues, and a photoperiod that mimics seasonal changes. Fish were fed once daily frozen bloodworms, brine shrimp, mysis shrimp, and cyclopeez and held at 22.2°C.

Eggs were stripped by hand from 13 females. On average there were 73.2 ± 9.0 eggs per clutch (mean $\pm$ standard error) and eggs averaged 2.69 ± 0.14 mg/egg. Clutches were divided into pools of 10 eggs/pool and placed into glass petri dishes with L-15 culture medium; culture medium was used in place of water to avoid the process of egg hardening that occurs when eggs are exposed to water (Tagawa et al 2000). The experiment used a within-clutch design; clutches were divided equally among concentrations with the constraint that all of the eggs from a particular female were used for either testosterone uptake or cortisol uptake.

Testosterone and cortisol were first dissolved in ethanol, then diluted into culture medium. Control medium contained the same amount of ethanol (maximum 0.05%). After one hour eggs were removed from the treatment, rinsed twice with 500 μ l L-15 medium, and frozen at -80°C. We performed the testosterone exposure (n=6 females) in May-June 2011 and the cortisol exposure (n=6 females) in May-July 2012.

Testosterone Radioimmunoassay. Testosterone concentrations were quantified via radioimmunoassay (RIA) (Wingfield and Farner 1975, Paitz and Bowden 2009). Briefly, samples (10 eggs each) were added to 1 ml of water containing 2000 cpm of tritiated testosterone and homogenized using a Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK, USA). Steroids were extracted twice with 3 ml of diethyl ether that was dried under nitrogen gas, reconstituted in 90% ethanol, and stored at -20°C overnight to precipitate neutral lipids. Testosterone was separated via column chromatography and quantified using competitive binding RIAs using antibody T 3003 (Wien Laboratories). Steroid concentrations were calculated based on a standard curve that ranged from 1.95 to 500 pg. Average recovery was 72% and the intra-assay

coefficient of variation was 7%. Tritiated testosterone (NET 553) was purchased from PerkinElmer (Boston, MA, USA).

Cortisol assay. Egg samples were thawed, homogenized using a battery-operated pestle grinder (Thermo Fisher Scientific, Hampton, NH), and centrifuged to isolate the supernatant. Samples were measured in duplicate using a cortisol competitive enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Enzo Life Sciences, Plymouth Meeting, PA). The antibody used in this assay is reported by the manufacturer to have a sensitivity of 56.72 pg/ml and a 27.7% cross reactivity with corticosterone, 4.0% with 11-deoxycortisol, 3.6% with progesterone, and 0.1% or less with testosterone, androstenedione, cortisone, and estradiol. Samples were diluted with assay buffer at ratios that allowed their expected concentration on the ELISA plate to fall within the standard curve. Diluted samples were divided in half to be measured as duplicates. To extend the manufacturer's recommended standard curve for the ELISA an 8th standard of cortisol (78 pg/mL) was included on each plate. Following the manufacturer's protocol, the plates were prepared and absorbance read at 405nm for each sample in duplicate on 96-well ELISA plates (Enzo Life Sciences, Plymouth Meeting, PA) on a FilterMax F3 microplate reader (Molecular Devices, Sunnyvale, CA) and absorbance data averaged across duplicate samples with Multi-Mode Analysis software (Molecular Devices version 3.4.0.25).

Blank-corrected, total-activity-corrected optical densities were converted to 'percent bound' for each sample by applying an equation derived from each plate's standard curve. A four-factor polynomial standard curve was produced for each ELISA plate and tested for accuracy in calculating cortisol concentration from 'percent bound' value of the known standards. Sample values were multiplied by their dilution factor to obtain the cortisol concentration in ng/mL of the original, undiluted egg samples. The intra-assay coefficients of variation (COV) for the two plates used in the experiment were 6.19% and 5.02 %, and the inter-assay COV was 1.18%.

Statistical analysis. Data on cortisol and testosterone levels in eggs were natural log-transformed to meet assumptions of normality. For both the testosterone and cortisol

experiments one mother contributed subsets for two out of three concentrations and the remaining mothers contributed at least one subset for all three concentrations. Multiple measurements of the same mother within a concentration (n=1 cortisol, n=4 testosterone mothers) were averaged prior to statistical testing. To control for the effect of mother on steroid levels in eggs we performed a repeated measures ANOVA within the proc mixed command of SAS (v9.3) for each steroid with mothers' identity as a random factor, concentrations as repeated measures within mothers, and natural log-transformed steroid concentration in pg/egg as the dependent variable. We used a general Satterthwaite approximation, assumed a compound symmetry covariance structure, and performed a comparison of least squares means between concentrations with Bonferroni adjustment.

Results

We identified nine studies on five fish species that met our criteria (Table 3.1). The duration of exposure to steroids was variable across studies, ranging from one hour to three days. Studies on fertilized eggs (e.g. McCormick 1999; McCormick and Nachaev 2002) tended to use longer exposure durations than studies on unfertilized eggs. Including duration in the "steroid exposure" did not change the relationship between exposure and uptake (results not shown). Concentrations of steroid applied were also variable, ranging from 0.5 pg/L to 150 mg/L. In all, five studies used radioimmunoassay to measure steroid concentrations, two studies used high performance liquid chromatography, and one study used enzyme-linked immunosorbent assay.

The literature review suggests that exposure of eggs to exogenous steroids increases steroid concentrations (Fig 3.1). The uptake of testosterone, cortisol, and estradiol occurred in proportion to the concentration applied. There was no obvious difference in uptake of steroids by fertilized and unfertilized eggs (Fig 3.1).

Exposing stickleback eggs to testosterone and cortisol increased their concentration (Fig 3.1, circled values; effect of testosterone treatment $F_{2,9,26}=30.23$, $p<0.0001$; effect of cortisol treatment $F_{2,10,3}=17.74$, $p=0.0005$). The baseline (i.e. control) level of testosterone in stickleback eggs in our experiment was 2.26 ± 0.75 ng/g, equivalent to 6.74 ± 2.54 pg/egg. This is lower than the baseline testosterone level of unfertilized eggs as reported in Coho salmon (120 ng/g, Feist et al 1990) but higher than

in Eurasian perch (1.5 ng/g, Rougeot et al 2007) and medaka (2 to 3 pg/egg, Iwamatsu et al 2005). The baseline level of cortisol in stickleback eggs in our experiment was 11.80 ± 1.80 ng/g, equivalent to 27.12 ± 5.02 pg/egg. This concentration is lower than baseline cortisol levels reported for unfertilized eggs of brown trout (46.2 ng/g, Sloman 2010), tilapia (48 pg/egg, Tagawa et al 2000), and rainbow trout (3.2 ng/egg, Li et al 2010) and higher than the baseline reported in unfertilized stickleback eggs in another study (1 to 2 ng/g, Giesing et al 2011).

Exogenous exposure of stickleback eggs to both the ‘low’ and ‘high’ concentrations of testosterone significantly elevated testosterone levels in stickleback eggs (Fig 3.2a), corresponding to a 649% and 2619% increase over baseline, respectively. Exogenous exposure to cortisol elevated cortisol levels in stickleback eggs at the ‘high’ concentration but not the ‘low’ concentration (Fig 3.2b). Eggs from the “high” cortisol concentration had a 2787% increase over baseline cortisol. Though not statistically significant eggs from the “low” cortisol concentration had a 235% increase over baseline cortisol. An endogenous increase in cortisol previously observed in stickleback eggs after maternal exposure to a predator was 35% (Giesing et al 2011).

Discussion

Altogether, our results indicate that steroid hormones are taken up by fish eggs via simple diffusion, a feature thought to be a fundamental mechanism in the transmission of a mother’s experiences to offspring (as in birds; reviewed in Groothuis and Schwabl 2008). Indeed a positive correlation between levels of stress-induced maternal plasma cortisol and egg cortisol has been reported in fish (Stratholt et al 1997). These results also illustrate the utility of immersing eggs in steroids to increase egg steroid levels, an experimental procedure useful for researchers that seek to obtain specified steroid levels.

Although these results suggest that developing eggs can be exposed to maternal steroids via diffusion, there are other mechanisms in vivo that might mitigate egg exposure to maternal steroids. For example, a recent study in rainbow trout showed that follicular metabolism within females can limit the exposure of developing fish eggs to cortisol (Li et al 2012).

It has been suggested that the water-hardening that occurs in conjunction with fertilization to prevent polyspermy (reviewed in Coward et al 2002) acts as a barrier to diffusion of steroids and EDCs, thereby causing fertilized fish eggs to take up less steroid than unfertilized eggs. However, visual inspection of the data suggests that rates of uptake by fertilized and unfertilized eggs are similar, and that fertilized eggs maintain the ability to take up steroids. This suggests that eggs remain permeable to steroids after water hardening of the chorion. At least one implication of this finding is that eggs could still be exposed to steroids or endocrine-disrupting chemicals in the environment even after fertilization.

Many of the studies reviewed in this paper elevated egg steroid content beyond physiologically-relevant levels. Our results suggest that concentrations ranging from 10 to 15 ug/L are sufficient to elevate egg steroid levels to physiologically and ecologically relevant levels. For example, to obtain egg steroid levels that reflect naturally-occurring levels in sticklebacks we recommend doses below 10 ug/L. The immersion technique we describe here makes fish an excellent model for the study of hormonally-mediated maternal effects.

CHAPTER 4: PROGRAMMING OF THE EARLY EMBRYONIC TRANSCRIPTOME BY MATERNAL STRESS IN THREESPINE STICKLEBACKS

Abstract

There is growing evidence for maternal effects on offspring traits such as size, metabolism, behavior, and the stress response. For example, threespine stickleback (*Gasterosteus aculeatus*) females exposed to predation risk produced eggs with higher concentrations of cortisol and offspring that had an altered physiological stress response to predators. However, the molecular mechanisms by which mothers' experiences influence offspring traits are not well understood, especially in species that do not exhibit maternal care. Recent evidence suggests that exposure of offspring to maternally-derived hormones, RNAs or other factors during development alters the level of transcription of certain genes in the embryo, leading to an altered phenotype in adult offspring. However, the magnitude and nature of genome-wide transcriptional change in vertebrate embryos due to maternal stress is currently unknown. Therefore we investigated the effect of maternal exposure to predation risk on the embryonic transcriptome in stickleback. Using RNA-sequencing we compared genome-wide transcription in three day post-fertilization embryos of predator-exposed and control mothers. This developmental period is a time when maternal mRNAs are likely to be fully degraded and when the offspring brain and hypothalamus-pituitary-interrenal (HPI) axis are forming. We report the embryonic genes and biological pathways influenced by maternal exposure to predation risk, including many involved in metabolism, epigenetic inheritance, and neural differentiation. The connection between observed expression patterns and previously observed maternal effects on behavior and physiology, including organization and activation of the HPI axis, are discussed.

Introduction

Non-genetic maternal effects, or the influence of a mother's phenotype on her offsprings' phenotype, are widespread across taxa (Agrawal 2002; Fox et al 1999; Kaplan 1992;

Sheriff et al 2009). Mothers can affect offspring phenotype by the level of care they give (Champagne et al 2003), and the resources they provide (Badyaev et al 2007), including transfer of steroids to developing offspring (Jobe et al 1998). These maternal effects can be maladaptive (Kaplan 1992; human disease: Barker 1995; Sie et al 2011) or adaptive (Fox et al 1999; Storm and Lima 2010). However, we know little about the mechanisms by which mothers transmit information about the environment to offspring or how this information, once received by offspring, influences their phenotype non-genetically.

Studies on diverse organisms have shown that maternal stress can have long-lasting effects on offspring traits including survival (Price et al 1992; Saino *et al.* 2005; Gagliano & McCormick 2009; Sheriff *et al.* 2009), growth (McCormick 2006; McCormick 2009; Sheriff *et al.* 2009), morphology (Saino *et al.* 2005; Gagliano & McCormick 2009; Sheriff *et al.* 2009), and behavior (monkeys, Clarke & Schneider 1993; rats, Nishio et al. 2001; skinks, Shine & Downes 1999). Prenatal exposure to maternally-derived stress hormones is commonly regarded as a mechanism; exposure is known to have lifelong consequences if it occurs during sensitive periods in development when the HPA (hypothalamic-pituitary-adrenal) axis, or in fishes, HPI (hypothalamic-pituitary-interrenal) axis is developing (reviewed in: Weinstock 2008; Matthew & Phillips 2010; Jobe et al 1998; Hu et al 2008). If prenatal exposure to stress hormones has organizational effects, that is, if maternal hormones influence differentiation of the developing brain (Phoenix et al 1959; Arnold and Breedlove 1985; Arnold 2009), then early exposure to maternally-derived stress hormones might impact an individual's stress response for life (e.g. Green *et al.* 2011; Clarke et al 1994).

Predators are one of the most important naturally-occurring stressors for animals in natural populations (Canoine et al 2002; Blanchard et al 1998), and predator-induced maternal effects with life-long consequences for offspring have been documented in diverse taxa. The phenomenon affects diverse traits in offspring (Shine and Downes 1999; Storm and Lima 2010; Agrawal et al. 1999; Gilbert 1966; Weisser et al. 1999), including the glucocorticoid response to stressors (Sheriff et al 2009). For example, predator exposure is known to trigger the release of the glucocorticoid cortisol in adult sticklebacks (Bell et al. 2007) and its accumulation in their eggs, corresponding to a maternal effect on offspring antipredator behavior (Giesing et al 2011). In a previous

study on sticklebacks we showed that adult offspring of mothers exposed to a predator had an altered cortisol response when they experienced a predator (Mommer and Bell 2013). These findings suggest that maternally-derived cortisol diffuses into eggs, influences development of the offspring brain and HPI axis via early embryo exposure, and results in adult offspring with altered physiological and behavioral stress phenotype.

We know little, however, about the molecular mechanisms by which this maternal effect occurs. Genome-wide expression analysis is a promising approach to address this question: it is an open-ended and unbiased approach and can identify molecular mechanisms that respond to maternal experience that are currently unknown. Surprisingly, few studies have measured the transcriptomes of early embryos in fish or other organisms (but see Mathavan et al 2005), and as far as we are aware none have measured genome-wide expression in response to maternal stress. However, there are a handful of studies that have exposed embryos or eggs to exogenous hormones (cortisol, Li et al 2010; estrogen, Chandrasekar et al 2010; triiodothyronine, Walpita et al 2007, Liu et al 2000) or hormone-disrupting toxins (17 alpha-ethinylestradiol, Vosges et al 2010; bisphenol A, Aluru et al 2010) and measured the expression of particular candidate genes, for example insulin-like growth factor (Aluru et al 2010; Li et al 2010), growth hormone (Aluru et al 2010; Li et al 2010), various hormone receptors (estrogen receptors, Chandrasekar et al 2010; thyroid hormone receptors, Li et al 2010, Walpita et al 2007, Liu et al 2000; growth hormone receptors, Aluru et al 2010; Li et al 2010), and the steroid-processing enzyme aromatase (Vosges et al 2010). Giesing et al (2011) found that maternally-stressed stickleback embryos had higher initial respiration, suggesting increased yolk metabolism by these offspring. This suggests that maternal stress might also affect transcription of yolk-processing enzymes (Kwon et al 2001). Several studies have found a significant effect of maternal treatment on brain-specific gene expression in adult rodent and teleost fish offspring (serotonin, Mueller and Bale 2008; ACTH, Leatherland et al 2010; glucocorticoid receptor, Szyf et al 2005; estrogen receptor, Leatherland et al 2010). We expect that similar gene expression differences might appear three days post-fertilization in sticklebacks when the brain is forming (Mueller and Wullimann 2005; Swarup 1959).

This study seeks to uncover some of the molecular mechanisms by which the maternal effect on the physiological stress response occurs in threespine sticklebacks. Using RNA-sequencing technology we obtain the stickleback embryonic transcriptome for embryos of mothers exposed to predation risk and control mothers. Transcriptome data are then compared between maternal treatments to identify which biological functions and genes are differentially regulated in early embryos as a result of maternal stress, and how they are connected to HPI and neural development.

Methods

Animal collection and parental treatment

Juvenile threespine sticklebacks were caught by baited trap from the Navarro River, CA in July, 2011 and transported to our laboratory at the University of Illinois (Urbana, IL). Fish were housed until adulthood (approximately 1 year of age) in 37L tanks (53L x 33W x 24H cm) with gravel-bottom, artificial plant refugia, a flow-through system with UV, charcoal, particulate and biological filters that remove olfactory cues and a photoperiod that mimics seasonal changes at a density of 10 fish/tank. Fish were fed *ad libitum* once daily frozen bloodworms, brine shrimp, mysis shrimp, and cyclopeez. For identification throughout the experiment all individuals were injected once under the skin laterally to the dorsal spines with a unique pattern of elastomer which is visible only under ultraviolet light.

In August, 2012 when sticklebacks showed reproductive morphology (red throat, gravidity) they were separated by sex. Males were housed individually in 9L tanks, females were housed in groups of four in 26.5L tanks (36L x 33W x 24H cm) with opaque external shading and four fish per tank. Tanks of females were randomly assigned as control or predator exposed. The females in predator exposed tanks were chased daily for 30 seconds at a randomly chosen time with a rubber model of a sculpin (SL: 7cm) to simulate predation risk. Sculpin are a known stickleback egg predator in the Navarro river (personal observation). Females were chased 29.4 ± 7.3 days (mean $\pm$ standard error). Males and control females were not chased.

Rearing embryos

When females became visibly gravid they were squeezed to obtain eggs. Testes were dissected from individual males, macerated, and the suspension was used to fertilize eggs in a glass petri dish. Egg fertilization was confirmed by observation under a light microscope, where after eggs were transferred to mesh-bottom cups in 9L tanks with air bubblers directly beneath developing clutches. All embryo tanks were treated with 100uL of methylene blue solution to prevent fungal infection. A single clutch was obtained from n=8 control and n=8 predator-exposed females. Embryos were allowed to develop for 72 hours during which time unfertilized and infected eggs were removed once daily. We chose three days post-fertilization (dpf) because in zebrafish it is the earliest period when maternally-derived RNAs are thought to be fully degraded (Mathavan et al 2005) and when brain regions begin to develop in zebrafish (Mueller and Wullimann 2005). By 72 hpf embryos had pigmented eyes, melanopores, and intermittent tail spasms (Swarup 1956). Embryos were removed from incubation after 72.040 ± 0.012 (mean $\pm$ standard error) hours, placed in RNAlater RNA stabilization reagent for one hour, and stored at -80C until total RNA extraction in February, 2013 (frozen for 4 to 5 months). All embryos used in RNA-sequencing were produced between August and October, 2012.

RNA-seq

To obtain sufficient quantity of RNA to meet the needs of quantitation, quality control, and serve as template for cDNA library creation, we selected ten embryos from each mother (n=8 control and n=8 experimental mothers, n=160 embryos total) at random and pooled them. Pools represent independent mothers and so are treated as biological replicates. Embryo pools were homogenized and total RNA extracted from the homogenate using the AllPrep DNA/RNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (as in Triant and Whitehead 2009). Briefly, each embryo pool was flash-frozen on dry ice in a microcentrifuge tube, disrupted with a pestle, treated with extraction buffer containing β -mercaptoethanol, and then homogenized with a pestle using a battery operated pestle grinder (Fisher Scientific, Fair Lawn, NJ). Total RNA was purified and treated with on-column DNase digestion (Qiagen). RNA was quantified on a Nanodrop spectrophotometer (Thermo Scientific,

Hudson, NH) and quality verified on an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). The amount of total RNA obtained from embryo pools was 5.19 ± 0.49 (mean $\pm$ standard error) micrograms per sample (i.e. per ten pooled embryos).

cDNA libraries were constructed for all samples from 1ug total RNA using the TruSeq Stranded RNAseq Sample Prep kit (Illumina Inc., San Diego, CA), quantitated on a Qubit fluorometer (Life Technologies, Grand Island, NY), run on an Agilent bioanalyzer chip, diluted to 10nM, and quantitated again with qPCR. The library construction process involved selection of mRNA. Each of the 16 samples was individually barcoded. All samples were then combined into a single pool and the pool was divided in half and run on two lanes for 101 cycles on an Illumina HiSeq 2000 using a TruSeq SBS sequencing kit (version 3). The reads were single-end and 100nt in length. RNA-sequencing produced over 356 million reads with average quality scores over 30; there were 22.28 ± 0.48 million reads (mean $\pm$ standard error) per sample.

Genome alignment and differential expression analysis

Reads were aligned to the *Gasterosteus aculeatus* reference genome (BROADS1, Ensembl database version 71.1, Feb 2006) using TopHat 2 (version 2.0.8) and reads that were expressed at very low level filtered out (described below). Across all samples 88.95 ± 0.18 percent (mean $\pm$ stderr) of reads mapped to the genome, and 51.87 ± 0.79 percent (mean $\pm$ stderr) of reads that mapped to the genome mapped uniquely within known or predicted genes, consistent with other RNA-seq studies that used genomes with comparable assembly and annotation quality (Blow 2009). Filtering removed 1677 genes that did not have any counts in any sample and 3339 genes that did not have at least 1 count per million mapped reads in at least 1 sample. The remaining 17440 out of the 22456 genes in the stickleback genome representing aligned reads were evaluated for differential expression between maternally-stressed and control embryos using the exactTest function in EdgeR (bioconductor.org, version 2.11) and simultaneously using Cufflinks/Cuffdiff (version 2.1.1).

The process generated a gene list from EdgeR (n=295 genes, FDR cutoff = 0.10) and a gene list from Cuffdiff (n=268 genes, FDR cutoff = 0.05), which were compared for overlap and then combined into a single dataset (Figure 4.1; n=455 genes, hereafter

referred to as the “dataset”). Cuffdiff employs an algorithm that probabilistically divides ambiguous fragments among genomic loci and then counts expression data at the transcript level, resulting in counts of Fragments Per Kilobase of exon per Million mapped reads (FPKM, Trapnell et al 2010; Blow 2009). EdgeR uses a different algorithm that counts reads at the gene level, applies a normalization factor that adjusts for library size, and reports the results as the logarithm of modified Counts Per Million mapped reads (logCPM, Robinson et al 2010; Blow 2009). We used different FDR cutoff values for the two methods in order to offset the different algorithmic stringencies and obtain two lists with a comparable number of genes for each count method (Fig 4.1). The majority of genes falling between the 0.05 and 0.10 FDR thresholds in EdgeR are also significant within the Cuffdiff $FDR \leq 0.05$ threshold or they code for a different subunit of a gene that falls within the Cuffdiff threshold, confirming that our choice to use different FDR cutoffs did not greatly affect the composition of the gene list.

Stickleback Ensembl gene IDs were automatically pulled from BioMart (toplevel.fa version 70 ensemble.org) for all 455 genes along with 292 of the corresponding gene names; 100 gene names for the remaining Ensembl IDs were obtained manually from the Ensembl database (release 71, Apr 2013) based on 1-to-1 orthology to other vertebrate species, for a total of 393 genes (~86% of the dataset) with gene names. To visualize the general relationships between sample genome-wide expression levels we used the plotPCA command in R to visualize the clustering of samples on the first and second principle components.

GO/pathway analysis

To identify enriched gene ontology (GO) categories the list of 393 gene names was analyzed with the DAVID Functional Annotation Tool (v6.7; Huang et al 2009a,b) using the gene annotations and background genome of *H. sapiens*. A total of n=317 of the gene names (~81%) were successfully recognized by DAVID. GO annotations representing biological processes (GOTERM_BP_FAT), cellular components (GOTERM_CC_FAT), molecular functions (GOTERM_MF_FAT), and KEGG pathways were considered significant if their false discovery rate (FDR) did not exceed a 0.05 threshold. To test the reliability of using genome as background the DAVID analysis

was rerun using Human Ensembl IDs corresponding to 15504 of the 17440 detected stickleback genes (~89%). The majority of the 317 genes from the gene list (>80%) were not represented in the output when Human Ensembl IDs converted from the list of detected stickleback genes were used as background, likely due to incomplete annotation of genes with official gene symbols within DAVID. Using detected (i.e. expressed) genes as background reduced the total number of terms that met the FDR threshold but did not change the nature of the terms obtained (data not shown). To test the effect of species-specific quality of annotation within DAVID the analysis was also run using mouse annotations and mouse genome background: 294 of 455 genes from the gene list (64.6%) had corresponding gene symbols within DAVID. The analysis was run again using zebrafish annotations and zebrafish genome as background: 165 of 455 DE genes (36.3%) had corresponding gene symbols within DAVID. Finally a random list of n=455 genes were selected at random from list of 17440 expressed genes in stickleback embryos and run through DAVID to identify quantity and type of results that are obtained by chance. This was repeated three times using human annotations and genome as background. In all 71-75% of genes in the lists were mapped within DAVID, and all three trials produced no significant GO_BP, GO_CC, GO_MF, or KEGG pathways below the FDR=0.05 cutoff.

A separate functional classification was performed using Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Redwood City, CA) using Human Ensembl Gene IDs converted from the 455 Stickleback Ensembl Gene IDs in BioMart. Because the human genome has multiple orthologs per gene for many stickleback genes, the number of Human Ensembl Gene IDs (n=596) was greater than the number of genes in the original dataset. There were corresponding Human Ensembl Gene IDs for n=302 of the genes in the dataset (~66%). Gene identifiers, their fold changes, and FDR values were uploaded to IPA and queried against the Ingenuity database to identify biological functions enriched in the dataset. A total of n=334 of the Human Ensembl Gene IDs (~56%) were successfully recognized by IPA.

Results

Overall expression patterns

The analysis produced $n=455$ genes differentially expressed between embryos from control and maternally-stressed mothers that were identified either by EdgeR, Cuffdiff, or both (Fig 4.1); nearly twice the number of genes were upregulated ($n=302$) than were downregulated ($n=153$). All of the genes that were identified as differentially expressed in both EdgeR and Cuffdiff ($n=108$) were expressed in the same direction between control and maternally-stressed offspring (i.e. the technique used to compare read counts between treatments did not influence their nature of gene expression difference), and there was a high correlation ($R^2=0.855$) between the magnitude of fold change (FC) identified using each method (Fig 4.2). In all there were 81 genes with greater than two-FC between treatments (22 downregulated and 59 upregulated in maternally-stressed offspring compared to control) and 32 genes with greater than five FC (5 downregulated, 27 upregulated).

Whole-genome expression data from each sample (a single clutch from $n=8$ control and $n=8$ predator-exposed mothers) were analyzed by PCA and show a general separation of maternal treatments along each of the first and second principle components (Fig 4.3), accounting for 18.7% and 12.0% of the variation, respectively. A heatmap comparing the expression patterns within and between samples shows a similar trend; roughly 2/3 of differentially-expressed genes are upregulated and 1/3 downregulated in embryos of predator-exposed mothers compared to embryos of control mothers (Fig 4.4).

GO enrichment analysis in DAVID revealed nine biological processes, seven cellular components, four molecular functions, and five KEGG pathways sufficiently enriched in the dataset ($FDR<0.05$) as compared to the human genome (Table 4.1a), including GO terms related to mitochondrial functioning, oxidative metabolism, neurological disease, and immune function. Changing the annotation and background from human to mouse and zebrafish tested whether the quality of between-species annotations within DAVID influenced the quality of GO and KEGG enrichment results. Using mouse annotations instead of human led to the inclusion of GO_BP terms associated with osmoregulation and coagulation, but did not largely change the GO_CC, GO_MF, or KEGG pathway annotations (Table 4.1b). Using zebrafish annotations

instead of human led to the inclusion of a new GO_BP term “generation of precursor metabolites and energy”, though this is not largely different from previously obtained GO_BP terms, and many fewer terms met the FDR threshold (Table 4.1c). The consistent recovery of GO and KEGG enrichment results across species, coupled with the failure of randomly-drawn gene lists to obtain any significant results, suggests that the results of the original DAVID analysis (Table 4.1a) are not expected by chance and are not heavily biased by the use of human annotations and genome background.

Pathway analysis in IPA revealed 13 clusters that represent cell proliferation, hematopoiesis, sensory organ development (especially eye development), adhesion of immune cells, kidney development, and metabolism of amino acids (Table 4.2). The PIANO gene set analysis using human orthologs of the DE genes in the dataset produced a network linking major GO annotations into a network, indicating the enrichment of several terms and connections including upregulation of protein binding and nucleus, and downregulation of small molecule metabolic genes and extracellular region (Fig 4.5).

HPI axis

One gene involved in HPI axis function, the FK506 binding protein 4 (FKBP4), was significantly downregulated in maternally stressed embryos (FC = -1.210, FDR = 0.073). Several genes central to HPI axis function were not expressed at all (i.e. all samples < 1 CPM), including corticotropin-releasing hormone (CRH), the CRH receptor 1 (CRHR1), one gene encoding proopiomelanocortin (POMC), the adrenocorticotrophic hormone receptor (MC2R), and one of the genes encoding 3- β -hydroxysteroid dehydrogenase (HSD3B2), which is involved in the de novo synthesis of cortisol. Others were expressed at low levels and were not significantly different between maternal treatments, including the second gene encoding POMC (logCPM=0.114), the genes encoding proprotein convertase 1 (PCSK1) (logCPM=0.353), steroidogenic acute regulatory protein (star) (logCPM=0.606), the cholesterol side-chain cleavage enzyme (CYP11A1) (logCPM= -0.22), steroid 17 α -monooxygenase (CYP17A1) (logCPM=0.850), and the CRH receptor 2 (CRHR2). Finally, several genes involved in the production of cortisol precursors, and the reception and metabolism of cortisol were expressed at meaningful levels but were not differentially between maternal treatments, including

proprotein convertase 2 (PCSK2) (logCPM=2.03), carboxypeptidase E (CPE) (logCPM=3.20), peptidyl-glycine alpha-amidating monooxygenase (PAM) (logCPM=4.08), lysosomal pro-x carboxypeptidase (PRCP) (logCPM=5.63), one of the genes encoding 3- β -hydroxysteroid dehydrogenase (HSD3B7) (logCPM=4.11), the genes encoding 11 β -hydroxysteroid dehydrogenase 2 (HSD11B2) (logCPM=5.57) and 3 (11-beta-hsd3) (logCPM=4.72), and the two genes that code for the glucocorticoid receptor (NR3C1) (logCPM=3.00 and 3.44).

Sexual maturation

Enzymes responsible for the metabolism of sex steroids were upregulated including steroid sulfatase (STS) (FC = 1.662, FDR = 0.094) and 17 β hydroxysteroid dehydrogenase 10 (HSD17B10) (FC = 1.233, FDR = 0.079). Gonadotropin-releasing hormone receptor 1 (GNRHR1), responsible for the reception of GnRH and subsequent release of LH and FSH during sexual maturation, was downregulated (FC = -3.023, FDR = 0.091). Also upregulated were the male sex-determining protein (DMRT1Y) (FC = 1.45, FDR = 0.032) and one gene encoding sex determining region Y-box 4 (SOX4) (FC = 1.66, FDR = 0.011).

Epigenetic machinery

We detected the differential expression of several genes involved in the maintenance of epigenetic modifications (Fig 4.6a), including DNA methyltransferase 3A (DNMT3A) (FC = -1.32, FDR = 0.078) and 3B (DNMT3B) (FC = -1.31, FDR = 0.055), the solute carrier family 46 member 3 (SLC46A3) (FC = -1.39, FDR = 0.021), two genes encoding the histone H1 protein (HISTH1A) (FCs = -2.30 and -1.48, FDR = 0.040 and 0.041), a gene encoding histone H2 (H2AFJ) (FC = 2.072, FDR = 0.094), the HIR histone cell cycle regulation defective homolog A (HIRA) (FC = -1.22, FDR = 0.095), and the histone protein transcriptional interacting factor DnaJ homolog, subfamily A member 1 (DNAJA1) (FC = -1.35, FDR = 0.007) and member 2 (DNAJA2) (FC = -1.18, FDR = 0.095).

Neural organization

Several genes involved in the differentiation of neurons and the formation and survival of neurites were differentially expressed between maternal treatments (Fig 4.6b). These included upregulation of two genes that encode cerebellin 9 (cbln9) (FCs = 1.82 and 1.53, FDRs = 0.013 and 0.006), a gene encoding the fibroblast growth factor receptor 1 (FGFR1) (FC = 2.40, FDR = 0.003), ceruloplasmin (CP) (FC = 1.61, FDR = 0.050), and orthodenticle homeobox 2 (OTX2) (FC = 1.56, FDR = 0.006), and downregulation of quaking (QKI) (FC = -1.34, FDR = 0.018), drebrin (DBN1) (FC = -1.21, FDR = 0.086), and the microRNA miR-9a (ola-mir-9a-1) (FC = -2.58, FDR = 0.026). Genes involved in eye formation were also upregulated, including ten genes coding for gamma crystallin (crygm) (mean FC = 1.39, mean FDR = 0.028), and the genes encoding phakinin (BFSP2) (FC = 1.92, FDR = 0.033), beta-carotene 15,15,-monooxygenase 1 (BCMO1) (FC = 1.39, FDR = 0.033), and recoverin (RCVRN) (FC = 1.82, FDR = 0.018).

Metabolism

Several genes involved in oxygen transport, ATP synthesis, and other metabolic processes were significantly upregulated in maternally-stressed embryos including embryonic hemoglobin alpha (hbae1) (FCs = 5.09 and 2.34, FDRs = 0.006 and 0.055) and beta (hbbe1.1) (FC = 1.47, FDR = 0.090), all of the major cytochrome c oxidase subunits (COX1,2,3,4i2,5b,6a2,7c) (mean FC = 1.55, mean FDR = 0.041), cytochrome b (CYTB) (FC = 1.64, FDR = 0.008), lactate dehydrogenase D (LDHD) (FC = 1.29, FDR = 0.030), solute carrier family 25 member 1 (SLC25A1) (FC = 1.26, FDR = 0.093), ubiquinol-cytochrome c reductase, complex III subunit VII (UQCRQ) (FC = 1.36, FDR = 0.020), betaine--homocysteine S-methyltransferase (BHMT) (FC = 1.36, FDR = 0.080), ethanolamine kinase 1 (ETNK) (FC = 1.55, FDR = 0.055). The gene encoding thyroid hormone receptor interactor 13 (TRIP13) was downregulated (FC = -1.26, FDR = 0.094).

Immunity

Many genes involved in the innate and adaptive immune response were differentially expressed in maternally-stressed embryos. Four genes encoding complement component 3 (C3) were significantly downregulated in maternally-stressed

embryos (mean FC = -1.72, mean FDR = 0.006), but a different two C3 genes were upregulated (mean FC = 1.54, mean FDR = 0.006). Also upregulated were complement component 6 (C6) (1.57, 0.021), complement component 8B (C8B) (1.38, 0.044), cytokine-inducible SH2-containing protein (cish) (1.34, 0.084), MHC class II antigen (ORLA-DCB) (7.07, 0.069), tapasin (TAPBP) up innate (2.10, 0.063), and NFKB-inhibitor alpha (NFKBIA) (1.28, 0.093). Downregulated were chemokine C-X-C motif ligand 12 (CXCL12) (-1.34, 0.076), immunoglobulin heavy constant mu (IGHM) (-22.21, 0.049), and NLR family member X1 (NLRX1) (-2.88, 0.055).

Discussion

We found that, in general, maternal stress had an activating effect on genome-wide expression and affected biological pathways involved in energy homeostasis, proliferation of cells, production of neurites and blood cells, differentiation of sensory neurons, and immunity. Many of these findings are consistent with previously findings in sticklebacks and other animals that associated maternal stress with increased offspring growth (Jobe et al 1998; Li et al 2010) and metabolism (Giesing et al 2011), and altered immune function (Von Hertzen 2002) and behavior (Giesing et al 2011; McGhee et al 2012; Weinstock 2008). Our data suggest that when mothers are exposed to a stressor, a shift occurs at the molecular level in offspring, leading to a divergence in the developmental program. Since many of the physiological systems involved in the embryonic response to maternal stress are homologous between sticklebacks and mammals (Clarke & Schneider 1993; Nishio et al. 2001), amphibians (Hu et al 2008; Kaplan 1992), reptiles (Shine & Downes 1999), and birds (Marasco et al 2012; Groothuis and Schwabl 2008), it is likely that they are evolutionary conserved in vertebrates.

HPI axis

Many of the genes that are essential for the functioning of the vertebrate HPI axis are not expressed in 3dpf stickleback embryos, including several key neuroendocrine ligands, their receptors, and enzymes responsible for the production of cortisol. These results are not surprising given a recent finding in sticklebacks that the level of cortisol in embryos reaches a low point 3dpf (Paitz et al, in prep), which suggested that most

maternally-derived cortisol is either inactive or gone by then, and the HPI axis is not yet active. Importantly, though, we detected the expression of the glucocorticoid receptor (NR3C1). While its expression did not differ between maternal treatments, its presence, and the presence of several genes responsible for the de novo synthesis and metabolism of cortisol, suggests that the cellular targets of the HPI axis are active. Also, we detected a significant effect of maternal stress on the expression of genes shown to be similarly influenced in humans and other mammal cells by exposure to the synthetic glucocorticoid dexamethasone. For example retinoic acid receptor gamma (RARG) and complement component 3 (C3) were downregulated in our study and are also downregulated in certain tissues in response to dexamethasone, RARG in breast cancer cells (Wan et al 2002) and C3 in lens epithelial cells (James et al 2005). Similarly, several genes upregulated in our study are also upregulated in response to dexamethasone including superoxide dismutase 1 (SOD1) in the retina (Miyarna et al 2008), cytokine-inducible SH2-containing protein (cish) in T lymphocytes (Bianchi et al 2000), NFkB inhibitor alpha (NFKBIA) in the brain (Quan et al 2000), and insulin-like growth factor binding protein 2 (IGFBP2) in fetal tissue (Price et al 1992). It is therefore possible that some of the genes differentially expressed in stickleback embryos in response to maternal stress were activated or repressed through the action of maternal cortisol. Further study to determine the magnitude and tissue specificity of the influence of maternal cortisol on the embryonic transcriptome remains to be determined.

Sexual maturation

Surprisingly, there is evidence that gonadal steroids pathways are involved in the observed differential gene expression between maternal treatments. DMRT1Y and SOX4, genes coding for vertebrate male sex-determining proteins and HSD17B10, which codes for enzyme that converts androstenedione to testosterone, were upregulated in maternally-stressed embryos. It is important to note that neither DMRT1Y (ENSGACG00000018819) nor SOX4 (ENSGACG00000009053) are present in stickleback linkage group 19, the chromosomal region identified by Peichel et al (2004) to contain SOX6 (ENSGACG00000006649), the primary sex-determining gene in sticklebacks. It is possible that DMRT1Y and SOX4 are involved in secondary sex

differentiation or indirectly in primary sex differentiation, though this has not been determined in sticklebacks. Steroid sulfatase (STS), responsible for the generation of androgen precursors and the conversion of estrone sulfate to estrone (Purohit et al 2011) was upregulated, and gonadotropin-releasing hormone receptor 1 (GNRHR1), responsible for the reception of the GNRH signal leading to the release of sexual differentiation hormones, was downregulated in maternally stressed embryos. These results suggest that sexual maturation and gonadal differentiation could be occurring earlier in maternally-stressed embryos. Sticklebacks are external fertilizers and stickleback males are the heterogametic sex, so it is interesting that maternal stress would affect sexual differentiation when sex is not determined until after mothers have broken all contact with offspring.

It is possible that stressed stickleback mothers influence sexual differentiation via the diffusion of altered levels of gonadal hormones into eggs. Recent findings that stickleback eggs can absorb elevated testosterone from their surroundings (Mommer and Bell, unpublished) support the idea that elevated levels of gonadal hormones in a mother could potentially enter her eggs, though the influence of predator stress on stickleback mothers' levels of sex hormones has not been tested. Gonadal hormones are known to play a significant role in the sexual differentiation of the brain and behavior in vertebrates (Balthazart and Ball 1995; Dewing et al 2003), and sex-specific maternal effects are known to occur (Mueller and Bale 2008; Uller et al 2009), suggesting that male and female stickleback embryos could be differently influenced by maternal stress during development. Since we measured pools of embryos it is not possible to determine whether expression of these genes was sex-specific, though if there are sex differences in the expression of these genes the fact that we detected significant differences despite pooled sexes suggests that the magnitude of these differences is an underestimate.

Epigenetics

Several factors involved in the epigenetic silencing of gene transcription, including multiple DNA-methyltransferases and two histone proteins, were differentially expressed in maternally-stressed embryos. DNMT3 is responsible for de novo methylation, specifically methylation at CpG sites, during development (Okano et al

1999). Decreased methylation of CpG sites in or nearby genes is associated with increased transcription (Jaenisch and Bird 2003) and so downregulation of DNMT3A and DNMT3B, as we observed in this study, is expected to increase gene activation. Also downregulated in maternally-stressed embryos was the solute transporter SLC46A3, which is responsible for transporting folic acid across the membrane (Qiu et al 2006) to be used by cells as an important source of methyl groups for DNA methylation (Niculescu and Zeisel 2002). Further, we observed a downregulation of the histone H1 and H2A proteins, which are part of the histone complex responsible that interacts with methylated DNA to suppress transcription (reviewed in Jones et al 1998). Our observation of downregulation of these genome-wide epigenetic repressors of transcription are consistent with our finding that two times more genes were activated (i.e. upregulated) in response to maternal stress than were repressed (i.e. downregulated).

Neural development

We found an upregulation in maternally-stressed embryos of several genes involved in the growth, survival, and death of neurites, the differentiating axons and dendrites that newly-forming neurons use to contact one another (reviewed in Purves 2001). These include two separate genes encoding the cerebellin 9 protein (cbln9), which is involved in synapse formation of cortical neurons (Joo et al 2011), the gene ceruloplasmin (CP), involved in the balance between neuronal survival and death during inflammation (Glezer et al 2007), orthodenticle homeobox 2 (OTX2), which may play a role in sensory organ and brain development, including formation of the fore- and mid-brain and pituitary gland (Millet et al 1996), and fibroblast growth factor-receptor 1 (FGFR1), a gene that combines with neural cell adhesion molecules to stimulate the activity of FGFR and the growth of neurites (Saffell et al 1997).

We also detected upregulation of several genes involved in the formation and use of the eye. Several genes coding for gamma crystallin (crygm) were upregulated; crygm is involved in formation of the lens (Graw 1997) and potentially the formation of neurites (Fischer et al 2008). Phakinin (BFSP2), a protein expressed during fiber cell differentiation in vertebrate eye lens (De Longh et al 2001) was upregulated, as was recoverin (RCVRN), the protein responsible for regulating phosphorylation of rhodopsin

in the retina, specifically the recovery phase of visual excitation and adaptation to background light (Dizhoor et al 1991). Sanogo et al (2011) measured gene expression changes in the brains of adult sticklebacks exposed to predator cues and found differential regulation of several genes involved in photoreception and phototransduction. Our results suggest that, at the molecular level, the developing embryo visual system might respond in a similar way to maternal predator exposure that the adult visual system does to direct predator exposure.

Metabolism

We detected a significant effect of maternal stress on several genes involved in the metabolic processes, including upregulation of genes involved in the delivery of oxygen to tissues via embryonic hemoglobin alpha (hbae1) and beta (hbbe1.1), the production of ATP via all of the cytochrome c oxidase subunits (I-VII), anaerobic metabolism via lactate dehydrogenase (LDHD), and the metabolism of amino acids and lipids via the enzymes BHMT (Pajares and Pérez-Sala 2006) and ETNK (Vance and Vance 2004). Coupled with the upregulation of many genes involved in the increased proliferation of cells and a previous finding that maternally-stressed stickleback embryos are larger and consume more oxygen (Giesing et al 2011), these findings suggest that accelerated growth plays a large part in this maternal effect.

microRNAs

We identified several microRNAs differentially expressed between maternally stressed and control embryos including several small nucleolar RNAs (SNORA3,18,23,73,74, SNORD15, and SNOU85). Some snoRNAs are thought to regulate specific alternatively spliced transcripts (Kishore and Stamm 2006a,b), suggesting they could modulate the relative abundance of alternative transcripts of genes. Unfortunately the depth and type (i.e. single-end reads) of our RNA-sequencing does not allow for the identification of alternative transcripts. Future studies using paired-end reads and deep sequencing could shed more light on the role of snoRNAs in the differential regulation of the embryonic transcriptome resulting from maternal stress.

We also detected several novel differentially expressed miRNAs including one (ENSGACG00000022837) that was expressed more than 193 FC higher in maternally-stressed embryos than control embryos (FDR = 0.003). It is possible that some of these known or novel miRNAs are involved in the epigenetic transfer of maternal stress to offspring. For example an miRNA in zebrafish (miR-430) accelerates the deadenylation of maternal mRNAs during embryonic development, promoting their clearance from embryos (Giraldez et al 2006). Also, in mice, an miRNA (miR-124) is involved in the epigenetic control of embryonic growth rate (Grandjean et al 2009).

A recent study found that stress applied to male mice altered the abundance of specific miRNAs in their sperm and corresponded to a reprogramming of offspring glucocorticoid response to stress (Rodgers et al 2013). Rodgers et al (2013) identified that DNMT3A is a target of four miRNAs differentially expressed in response to paternal stress. In our study DNMT3A was significantly downregulated in maternally-stressed stickleback embryos. The presence of multiple miRNA target sites within a gene is thought to allow the fine-tuning of the expression of that gene between tissues (reviewed in Georges et al 2007). It is therefore possible that DNMT3A and one or more miRNAs are involved in an interplay between stress-induced maternal and paternal effects on the offspring stress response, and that DNMT3A expression might be parent- and tissue-specific in developing offspring.

We found that the microRNA miR-9a was significantly downregulated in stickleback embryos in response to maternal stress (fold-change = -2.58, FDR = 0.026). Loss of expression of miR-9a in *Drosophila* has been shown to increase the proliferation of sensory organ precursor cells and promote neuronal fate in nearby epithelial cells (Li et al 2006). miR-9a is also highly expressed in the mammalian brain and its nucleotide sequence is fully conserved between humans and *Drosophila* (Sempere et al 2004; Aravin et al 2003). The stickleback ortholog has 92% sequence identity to human miR-9a (megaBLAST alignment, $E=8e^{-26}$). It is therefore possible that the miR-9a downregulation we observed in maternally-stressed stickleback embryos functions to accelerate the proliferation and differentiation of sensory neurons.

Immunity

We detected the differential regulation of several immune genes, including genes involved in the innate and adaptive immune responses. Previous studies in humans (Wright et al 2010) and other primates (Coe et al 2007) have found an enhancing effect of maternal stress on the offspring innate immune response, coupled with a depression of the adaptive immune response (Von Hertzen 2002; Caserta et al 2008). Several of the genes involved in the complement pathway, which is responsible for the recognition and clearance of bacteria to prevent infection (reviewed in Abbas et al 2010), were upregulated in maternally-stressed stickleback embryos. Downregulated were the genes CXCL12, IGHM, and NLRX1 which are involved in the recognition and clearance of antigens by the adaptive immune response (Bleul et al 1996; O'Neill 2008; Abbas et al 2010).

Conclusion

In our study gene expression was measured in whole embryos. As a result, many tissue-specific expression changes resulting from maternal stress would not have been detected, particularly for genes that exhibit low levels of expression or are expressed in minute tissues. Similarly, we measured embryos only at one specific time point, 72 hours post-fertilization. With only one time point it was not possible to detect whether 72hpf represents a peak in the magnitude of difference between maternal treatments. Due to the understandably dynamic and rapidly-changing nature of genome-wide expression throughout development, it is likely that our data only represent a small piece of the puzzle. Future studies that measure tissue-specific embryonic expression or multiple time-points would likely obtain results in richer detail.

A maternal effect on the cortisol response to predation risk persists throughout the lifetime of stickleback offspring (Mommer and Bell 2013) despite the brief contact of stickleback mothers with their offspring, and the rapid metabolism of maternally-derived cortisol in embryos (Paitz et al, in prep). Our results suggest that 3dpf stickleback offspring respond to maternal predator exposure via increased neurite formation coupled with accelerated development of the visual system. Embryos also experience upregulation of many genes involved in aerobic and anaerobic metabolism, coupled with

a faster growth rate (Giesing et al 2011), suggesting that offspring of mothers exposed to a predator undergo a general acceleration of the developmental program, and as a consequence might be more likely to survive to reproduction in a high predation environment. Further study is needed to elucidate the myriad molecular interactions between genes that are differentially-regulated as a result of maternal stress and understand their biological and adaptive significance.

CONCLUSIONS AND FUTURE DIRECTIONS

Understanding how offspring react to the maternal environment shapes the way we understand societal problems such as the depressing effects of poverty on chronic disease (Miller et al 2009), the prevalence of drug and alcohol dependency (Diaz-Anzaldúa et al 2011), and the toxicity of substances we release into the environment (Fent and Sumpter 2011; Yang et al 2007). Much work has been done to identify the prevalence of maternal effects across taxa (Agrawal 2002; Fox et al 1999; Kaplan 1992; Sheriff et al 2009), but the mechanisms by which transgenerational transfer of information about the environment occurs is less understood (Chapter 1). For my dissertation work I sought to understand the mechanisms underlying the transfer of stressful experiences from mothers to offspring. First, I tested whether offspring of stickleback mothers exposed to a predator had an altered physiological stress response to predator exposure (Chapter 2; Mommer and Bell 2013). Next I reviewed the literature to understand whether fish eggs absorb stress (and other) hormones in an environment that simulates the internal environment of mothers, and I experimentally confirmed the relationship in sticklebacks (Chapter 3). Finally, I identified the changes in genome-wide expression of genes in early embryos that result from maternal exposure to a predator (Chapter 4). Here I discuss how these findings fit into the current scientific understanding of maternal effects and outline new questions and ideas that deserve further study.

Cortisol as a mechanism

There is a significant effect of maternal predator exposure on egg cortisol content (Giesing et al 2011) and the offspring cortisol (Mommer and Bell 2013) and behavioral (Giesing et al 2011; McGhee et al 2012; Roche et al 2012) responses to a predator. The presence of both elevated cortisol in eggs of stickleback mothers exposed to a predator and the effects of maternal predator exposure on offspring physiological and behavioral responsiveness suggests that cortisol affects the development of the brain or HPI axis in embryos. It is likely that activation of the HPI axis in female sticklebacks exposed to stressful events (Bell et al 2007) during oogenesis exposes her eggs to elevated cortisol and that her eggs take up some of that extra cortisol. I found that exogenous glucocorticoid and sex steroids diffuse into stickleback (and other fish) eggs (Chapter 3).

Together with studies that have shown exposing developing embryos to elevated cortisol affects the expression of physiological (Li et al 2010) and behavioral (Sloman 2010) traits confirms that the transfer of maternal steroids to eggs is a potential mechanism underlying the maternal effects observed in sticklebacks (Chapter 3). My results suggest that maternal steroids can enter fish eggs, but this leaves some assumptions untested. A recent study following the levels of four steroids through development in sticklebacks (Paitz et al, in prep) showed a precipitous drop by three days post-fertilization (dpf), suggesting that maternal steroids are probably quickly metabolized to their inactive forms by embryos. Whether the influence of maternally-derived steroids is restricted to the period prior to three dpf or whether embryos reactivate metabolites to their biologically active forms during development is currently unknown. The differential expression of steroid-reactivating enzymes in early embryos of predator-exposed mothers (Chapter 4) suggests that both early influence and later reactivation of maternal steroids might occur. A promising future direction is to track the levels of steroids and their metabolites throughout development in order to shed more light on the dynamic interaction between steroid production, transfer, and metabolism in stickleback mothers and their eggs.

The transcriptomic response to maternal stress

I found that the transcriptome of developing stickleback embryos is sensitive to maternal stress (Chapter 4). A comparison of genome-wide transcription patterns between embryos of mothers exposed to a predator and control embryos demonstrated that embryos respond to maternal stress via differential expression of pathways involved in metabolism, growth, epigenetics, neural development, sexual maturation, and microRNAs. The absence of expression of several genes responsible for HPI axis function suggests that the stickleback stress response is not yet active by three dpf, as recent data on the consistently low, unchanging level of cortisol during ontogeny in sticklebacks seems to confirm (Paitz et al, in prep).

Much of the evidence from this study suggests that embryos of mothers exposed to predators are experiencing accelerated growth and development, including primary sexual differentiation, perhaps at the expense of a well-developed adaptive immunity, longevity, or size at adulthood (Bell et al 2011). This correlates well with previous

findings showing elevated oxygen consumption in early embryos of sticklebacks (Giesing et al 2011) and increased growth in sticklebacks (Bell et al 2011) and other species (McCormick 2006; McCormick 2009; Sheriff *et al.* 2009; Li et al 2010, but see Jobe et al 1998) resulting from maternal exposure to predators. It indicates that offspring of predator-exposed mothers might complete development faster than control offspring, a potential advantage in high-predator environments (reviewed in Bell et al 2011). A study carefully measuring the effect of maternal predator exposure on the timing of specific stages of stickleback embryonic development, including date of hatching, would allow a more clear interpretation of these results.

Since I measured gene expression in whole embryo homogenates at one time point my data do not capture the temporal and spatial heterogeneity of gene expression that likely occurs in embryos. Future studies should aim to measure expression within and between tissues to identify possible regional heterogeneities in embryos that result from maternal stress, and at multiple developmental time points to identify when transcriptional events are differently regulated. Of particular interest is heterogeneity of expression in the brain, since it is the site of integration of environmental information (e.g. the presence of predators, conspecifics) into HPI and behavioral actions. Stickleback mothers are oviparous, externally-fertilizing, and do not exhibit maternal care, yet their experience with a predator affects the expression of several genes associated with neural development in developing embryos and the physiology and behavior of adult offspring. This indicates that the early vertebrate embryonic brain maintains a high sensitivity to hormonal and molecular signals that result from maternal stress. In other words prenatal exposure to maternal stress appears to have organizational effects, influencing differentiation of the developing brain (Phoenix et al 1959; Arnold and Breedlove 1985; Arnold 2009). These results suggest that the nervous system is being differently wired in embryos of mothers exposed to a predator, an effect that could be visualized via *in situ* hybridization of probes designed to detect specific differentially-expressed RNAs.

Adaptive vs maladaptive

The fact that maternal stress affected the offspring HPI response (Mommer and Bell 2013) suggests an altered ability of maternally-stressed offspring to recognize and

respond to external stressors. Whether that altered recognition and response allows offspring to better survive a live predator attack in the wild is a question that has not yet been answered in sticklebacks. Exposing stickleback to a live predator attack in the laboratory has shown that offspring of predator-exposed mothers survive for a shorter time than control offspring (McGhee et al 2012). Also, offspring of predator-exposed mothers exhibit slower learning in a color-discrimination task (Roche et al 2012). These studies suggest that the effects of maternal predator exposure are maladaptive for stickleback offspring, though it is important to note that both studies measured the behavior of stickleback offspring in the absence of a social group. In the wild sticklebacks can be seen swimming alone or in the presence of loosely-aggregated social groups. The presence of a social group affects both the physiology and behavior of sticklebacks: individuals have lower circulating cortisol when a group is present than when they are alone (Mommer and Bell 2013) and offspring of predator-exposed mothers swim more closely together than control offspring (Giesing et al 2011). It is therefore possible that, in sticklebacks, offspring of predator-exposed mothers are more likely to seek and benefit from the safety of a social group in an environment where predators are present. That is, the influence of maternal exposure to predators in sticklebacks could be maladaptive for offspring that are forced to be alone but adaptive if offspring are allowed to associate with conspecifics. Future studies wishing to elucidate the adaptive nature of this maternal effect in sticklebacks, whether in the field or lab, should attempt to incorporate the presence of a social group into the study design.

Maternal X paternal effects

Maternal microRNAs are known to be involved in mouse early embryonic development (Tang et al 2007) and have recently been implicated in a paternal effect in fish (Rodgers et al 2013). In my study (Chapter 4) many miRNAs were found to be differentially expressed in embryos of mothers exposed to a predator. Further, the promoter region of the gene encoding DNA methyltransferase 3A (DNMT3A) is known to have multiple miRNA binding sites and be differentially expressed based on paternal stress (Rodgers et al 2013). DNMT3A was differentially expressed in stickleback embryos exposed to maternal stress (Chapter 4). Together these results suggest that

miRNAs and DNMT3A might be involved in an interaction between maternal and paternal influence on transcript abundance in embryos. Future work looking at the patterns of expression of miRNAs and DNMT3A throughout development and spatially within developing embryos might shed more light on their role in mediating maternal and paternal effects. Also, it is possible that the expression of these genes is maternally- or paternally-imprinted, that is, the expressed allele in offspring could be primarily of maternal or paternal origin. If so, it would be interesting to see if different parental alleles are expressed in different brain regions in sticklebacks as has been observed in mice (Gregg et al 2010), and if miRNAs and DNMT3A are involved. Finally, recent evidence in zebrafish showed that an embryo-derived miRNA triggers the clearance of maternally-derived mRNAs from embryos (Giraldez et al 2006). It is possible that degradation of parental miRNAs (maternal or paternal), perhaps even in a tissue-specific pattern, is involved in epigenetic modification of gene expression through regulation of transcription of DNMT3A and other epigenetic machinery, thereby influencing gene- and tissue-specific expression through development. Further research is needed into the nature of miRNAs during development, their interaction with DNMTs and the abundance of maternally- and paternally-derived transcripts.

FIGURES AND TABLES

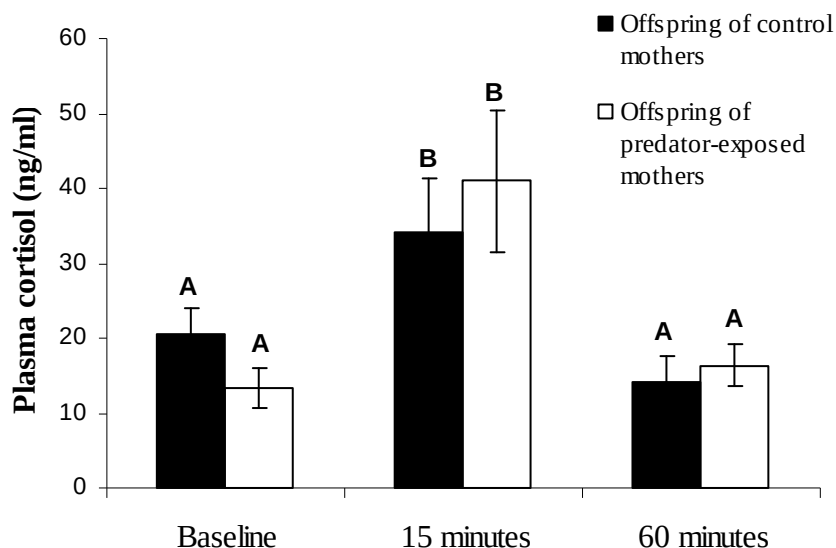


Figure 2.1, The cortisol response of offspring of mothers exposed to simulated predation risk compared to offspring of control mothers. There was a significant interaction between maternal treatment and time (Table 1), indicating that the time course of the plasma cortisol response differs between offspring of predator-exposed mothers and control mothers. However, the post-hoc comparison of least squares means between maternal treatments at each time point were not significant ($p > 0.05$). $N=28-30$ per bar. Data represent least squares (LS) means $\pm$ 1 standard error of raw (not transformed) data. LS-means with the same letter are not significantly different.

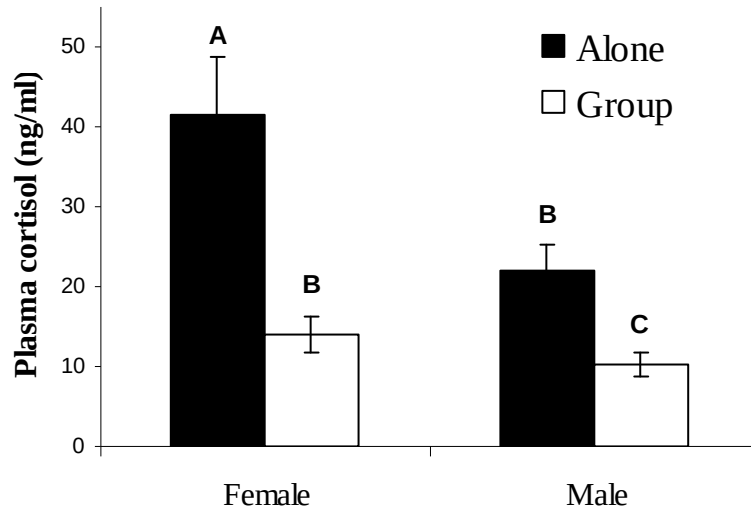


Figure 2.2, Sticklebacks that were alone had higher circulating concentrations of cortisol than sticklebacks that were in a group, and females had higher cortisol than males. Data within each level of sex*social environment represent all three time periods combined. N=43-51 per bar. Data represent least squares (LS) means $\pm$ 1 standard error of raw (not transformed) data. LS-means with the same letter are not significantly different.

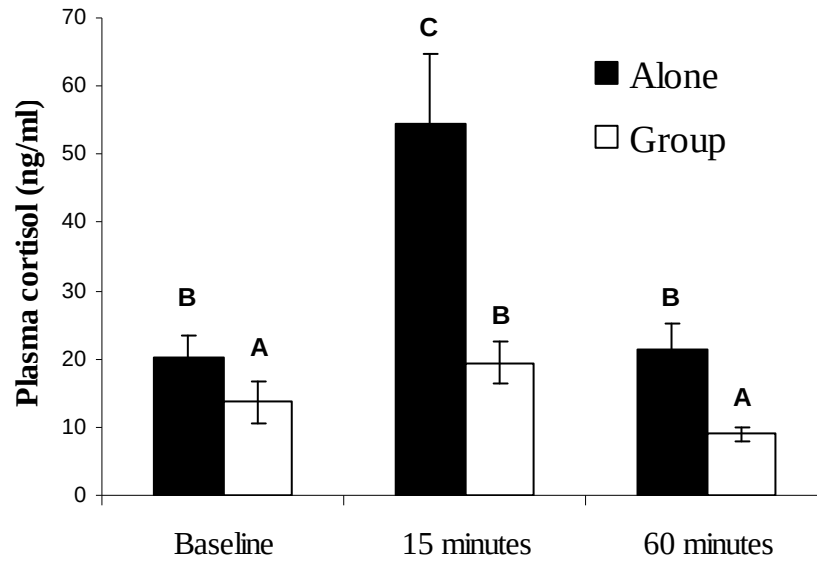


Figure 2.3, Levels of plasma cortisol increased 15 minutes after exposure to simulated predation risk and subsided by 60 minutes after exposure. Fish that were in a group consistently had lower plasma cortisol than fish that were alone. N=28-30 individuals per bar. Data represent least squares (LS) means ± 1 standard error of raw (not transformed) data. LS-means with the same letter are not significantly different.

Table 2.1, GLM model testing the effect of maternal treatment, social environment, sex, and exposure to predation risk over time on plasma cortisol in threespine sticklebacks. Date was included in the model as a covariate and did not significantly interact with any of the class variables (i.e. $p > 0.05$ for all interactions). Interactions that were not significant (i.e. $p > 0.05$) in the full model were stepwise-removed until only significant interactions remained.

Source	DF	Type I SS	Mean Square	F Value	P
Maternal treatment	1	0.07183249	0.07183249	0.10	0.7515
Time	2	22.41042515	11.20521258	15.69	<.0001
Social environment	1	22.04691896	22.04691896	30.87	<.0001
Sex	1	10.24221408	10.24221408	14.34	0.0002
Date	1	7.17508595	7.17508595	10.05	0.0018
Maternal treatment*Time	2	4.38936628	2.19468314	3.07	0.0490

Table 3.1, Data on the uptake of exogenous steroids in fish eggs from the literature. Shown are the species, whether the eggs were fertilized or not, the steroid applied, the concentration of the steroid applied, the duration of exposure, the control and post-exposure concentrations, the method of measurement. F=cortisol. E2=estradiol. T=testosterone. ELISA=enzyme-linked immunosorbent assay. RIA=radioimmunoassay. HPLC=high-performance liquid chromatography.

Species	Fert?	Steroid	Applied (ng/L)	Duration (hours)	Control (pg/egg)	Post-exposure (pg/egg)	Method	Reference
brown trout	NO	F	470220	3	2772.0	41940.0	ELISA	Sloman 2010
rainbow trout	NO	F	100000	3	3200.0	4700.0	RIA	Li et al 2010
rainbow trout	NO	F	1000000	3	3200.0	7200.0	RIA	Li et al 2010
tilapia	NO	F	5000	12	48.0	10.0	HPLC	Tagawa et al 2000
tilapia	NO	F	50000	12	48.0	100.0	HPLC	Tagawa et al 2000
tilapia	NO	F	500000	12	48.0	2000.0	HPLC	Tagawa et al 2000
tilapia	NO	F	5000000	12	48.0	20000.0	HPLC	Tagawa et al 2000
threespine stickleback	NO	F	10000	1	27.1	117.8	ELISA	this study
threespine stickleback	NO	F	500000	1	27.1	810.1	ELISA	this study
threespine stickleback	NO	T	10000	1	6.74	57.2	ELISA	this study
threespine stickleback	NO	T	500000	1	6.74	190.0	ELISA	this study
coho salmon	YES	E2	400000	2	264.0	2537246.0	HPLC	Piferrer and Donaldson 1994
medaka	YES	E2	1000	24	0.50	1.12	RIA	Iwamatsu et al 2005
medaka	YES	E2	3000	24	0.50	3.29	RIA	Iwamatsu et al 2005
medaka	YES	E2	10000	24	0.50	54.2	RIA	Iwamatsu et al 2005
medaka	YES	E2	100000	24	0.50	569.7	RIA	Iwamatsu et al 2005
medaka	YES	E2	500000	24	0.50	1675.5	RIA	Iwamatsu et al 2005
tropical damselfish	YES	F	1	72	0.35	0.80	RIA	McCormick and Nechaev 2002
tropical damselfish	YES	F	10	72	0.34	0.35	RIA	McCormick 1999
tropical damselfish	YES	F	100	72	0.34	0.71	RIA	McCormick 1999
tropical damselfish	YES	F	1000	72	0.34	0.79	RIA	McCormick 1999
tropical damselfish	YES	F	10000	72	0.34	1.12	RIA	McCormick 1999
brown trout	YES	F	200000	2	1100.0	5435.0	RIA	Burton et al 2011
tropical damselfish	YES	T	10	72	0.20	0.38	RIA	McCormick 1999
tropical damselfish	YES	T	100	72	0.20	0.80	RIA	McCormick 1999
tropical damselfish	YES	T	1000	72	0.20	3.05	RIA	McCormick 1999
brown trout	YES	T	200000	2	291.0	20130.0	RIA	Burton et al 2011
coho salmon	YES	T	400000	2	68.0	1794554.0	HPLC	Piferrer and Donaldson 1994

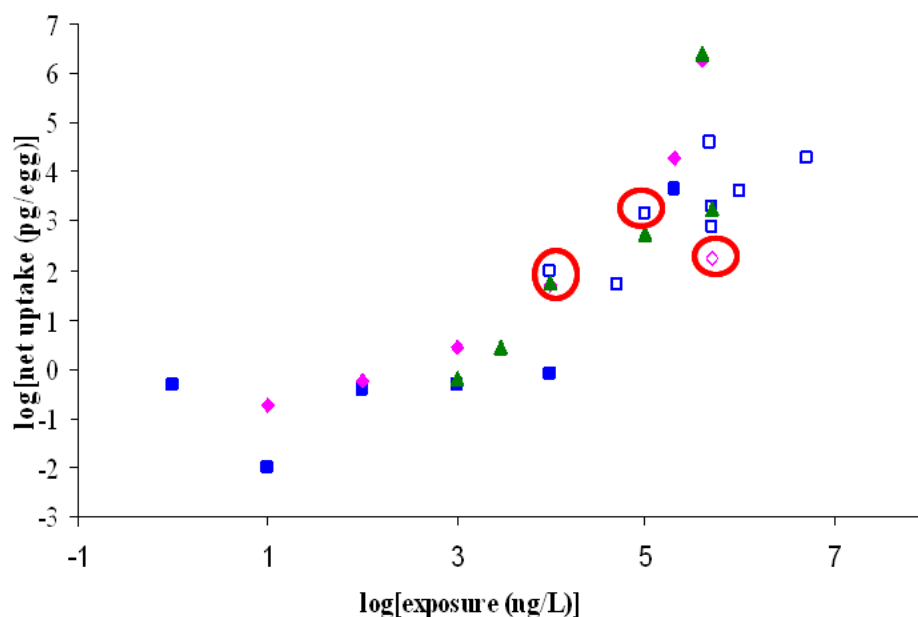
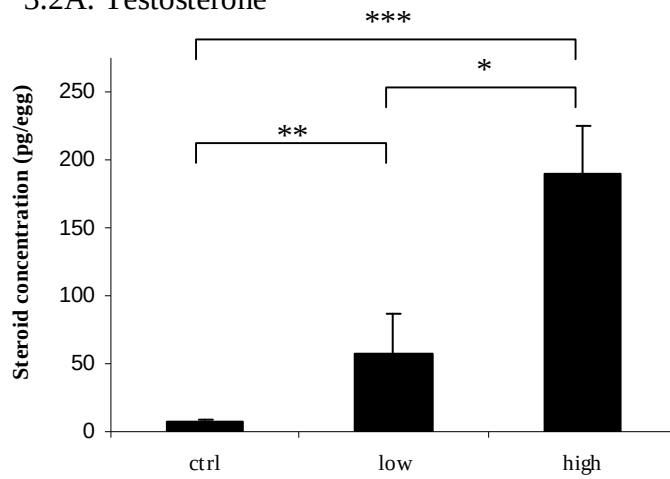


Figure 3.1. The uptake of cortisol (F, blue squares) testosterone (T, pink diamonds) and estradiol (E2, green triangles) by fertilized (closed symbols) and unfertilized (open symbols) fish eggs as reported in the literature. The amount of steroid in eggs after exposure increased with the concentration applied. Time and concentration of exposure were normalized to a common 'steroid exposure' term (nanograms steroid per liter of solution) and net uptake data were converted to picogram per egg. Circled values are data that we collected in our experiment applying testosterone and cortisol to unfertilized stickleback eggs. All data were log-transformed for ease of visualization.

3.2A: Testosterone



3.2B: Cortisol

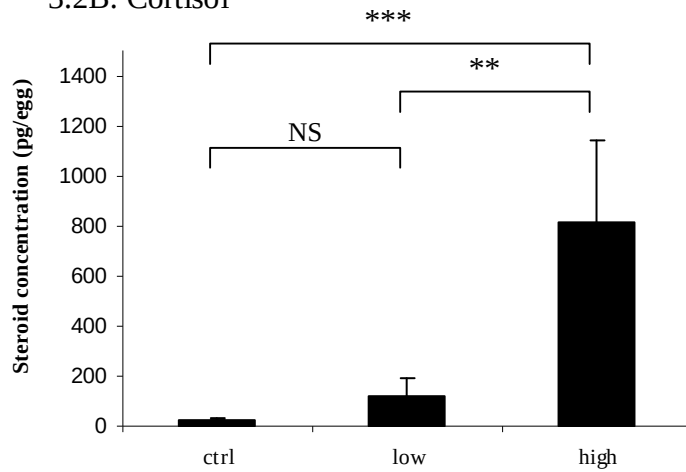


Figure 3.2, The concentration of A) testosterone and B) cortisol in unfertilized stickleback eggs in control (0 ug/L), low (10 ug/L), and high (500 ug/L) steroid concentrations. Each bar represents mean $\pm$ SE as measured in pools of eggs from n=6 mothers radioimmunoassay (testosterone) or enzyme-linked immunosorbent assay (cortisol). Levels of significance from Bonferroni-adjusted comparisons of least squares (LS) means were derived from a mixed model that controlled for repeated measurements within mothers; NS=adjusted $p > 0.05$, *adjusted $p < 0.05$, **adjusted $p < 0.01$, ***adjusted $p < 0.001$).

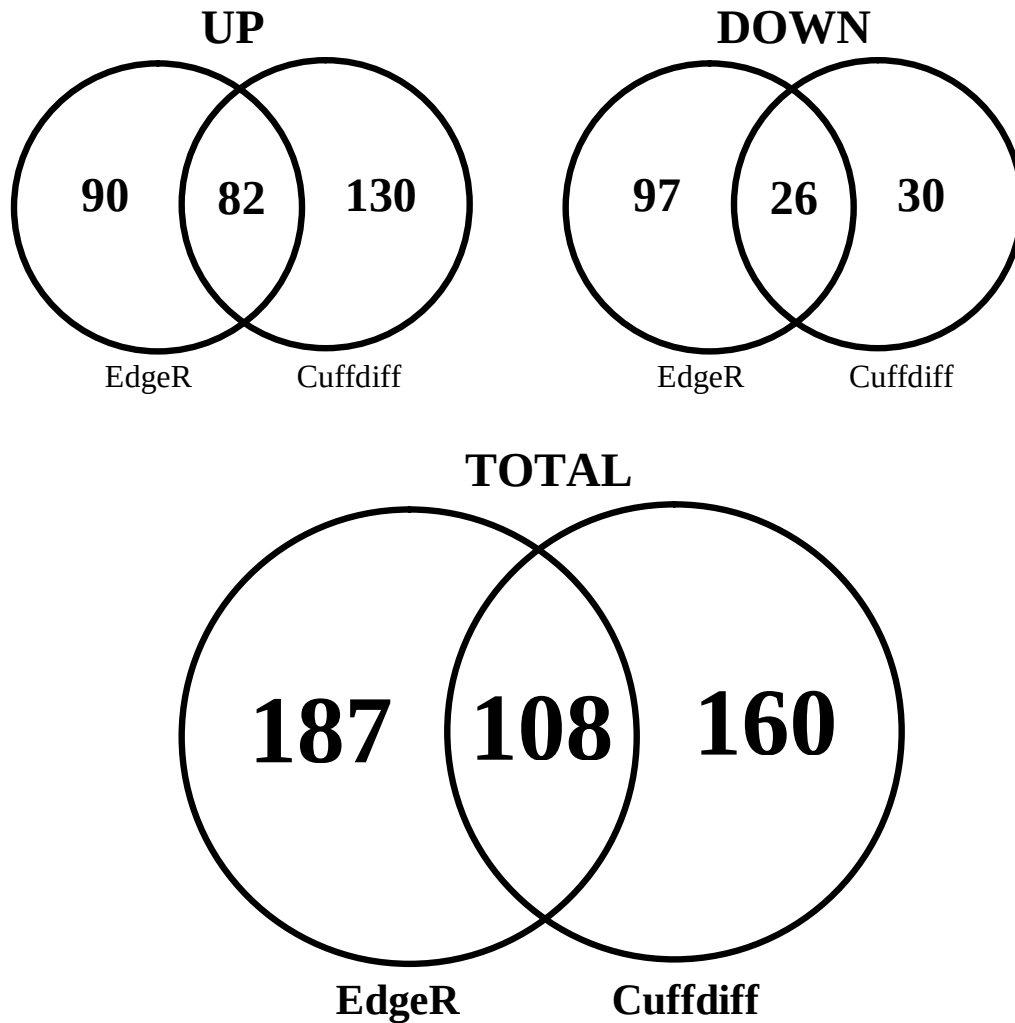


Figure 4.1, The intersection of upregulated, downregulated, and total differentially expressed genes as identified in EdgeR and Cuffdiff from the same alignment of reads to the stickleback reference genome (n=455 total unique genes, 302 upregulated, 153 downregulated).

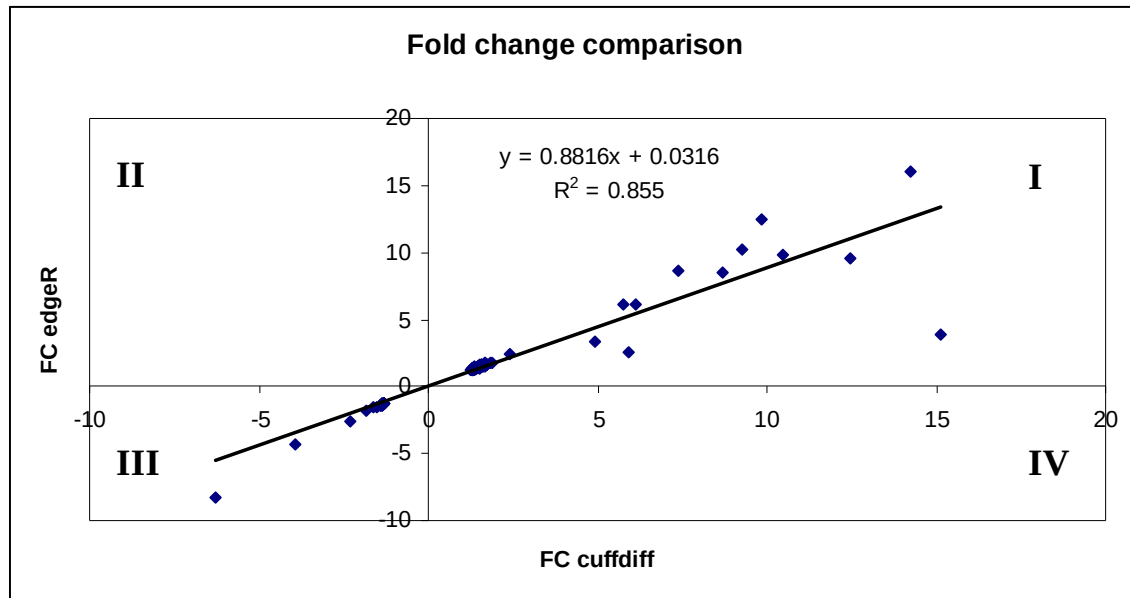


Figure 4.2, comparison of fold change data of $n=108$ genes identified via both Bowtie-edgeR and Cufflinks-Cuffdiff analyses as differentially expressed in stickleback embryos exposed to maternal stress. While Cuffdiff generally called a higher fold change than edgeR (slope = 0.88), there was nevertheless a tight correlation between the two (R -square = 0.855). All genes were found to be upregulated (quadrant I) or downregulated (quadrant III) in both analyses.

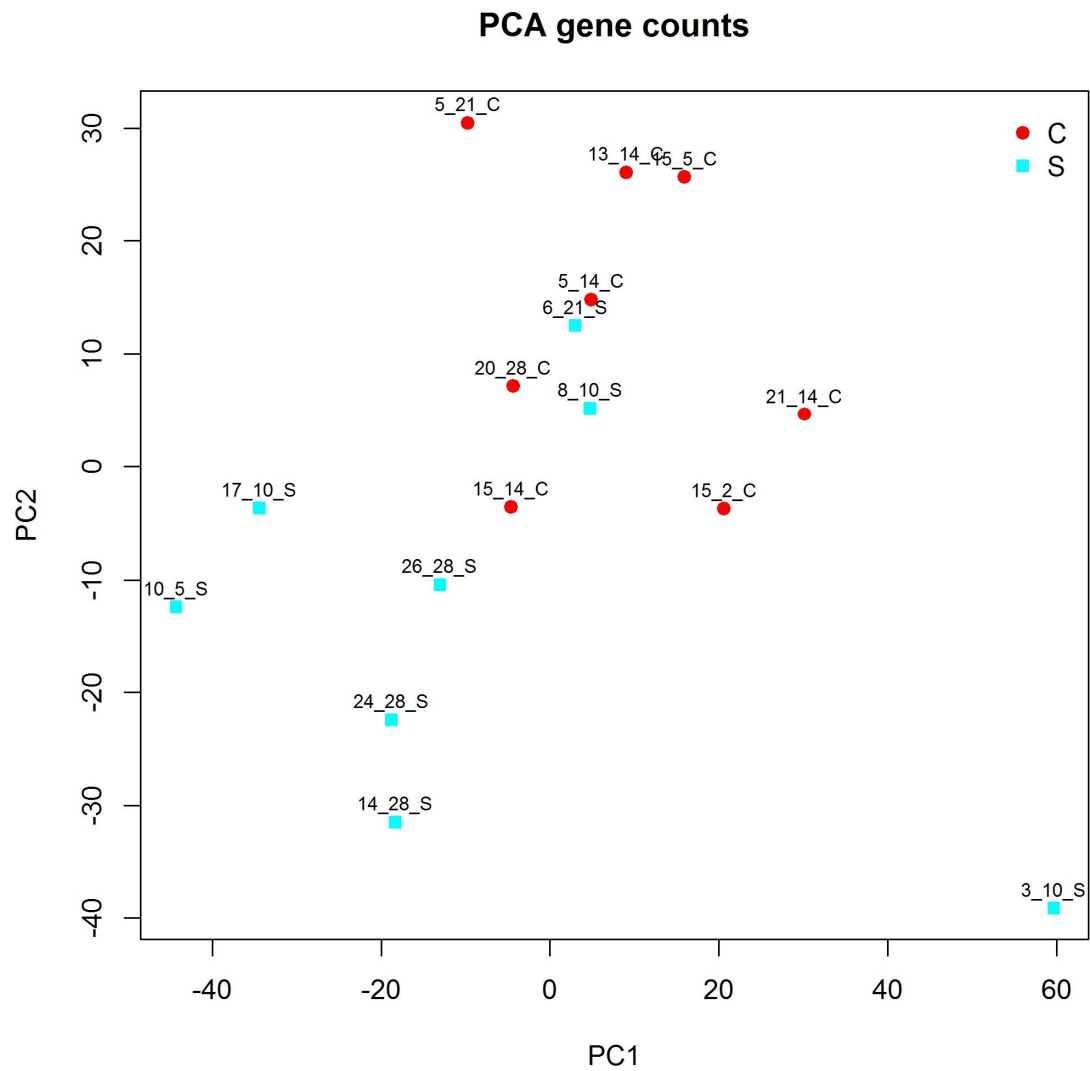


Figure 4.3, A Principle Components Analysis of general gene expression patterns of each sample as determined by RNA-seq and edgeR differential expression analysis. Blue squares represent embryos of mothers exposed to predation risk and red circles represent embryos of control mothers. Each data point represents total RNA from 10 pooled embryos from a single mother.

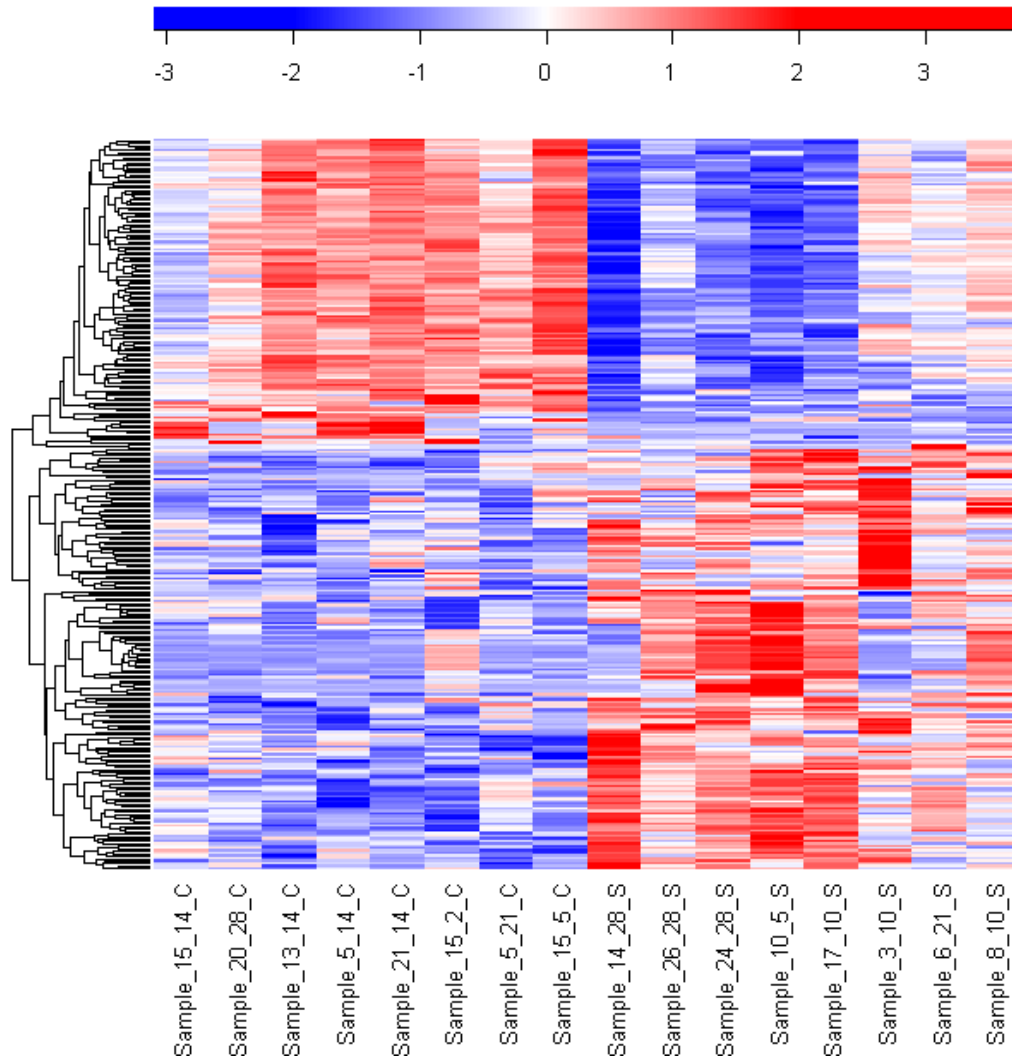


Figure 4.4. A heatmap showing the general pattern of gene regulation for the 295 genes found to be differentially expressed (EdgeR) between embryos of predator-exposed and control mothers. Red = upregulated, blue = downregulated compared to the mean value of a gene from all samples. Rows represent genes and columns represent clutches (one clutch per mother). C=control and S=maternally-stressed clutches. log(CPM) data are normalized for library size and scaled so that every gene has the same mean (0) and standard deviation; units of change are standard deviations from the mean, and each row has the same range of color values.

Table 4.1a, The most overrepresented biological annotations from the list of genes differentially expressed between 3dpf stickleback embryos exposed to maternal stress and embryos of control mothers as measured via RNA-seq. Genes were mapped to their associated biological process (BP_FAT), cellular component (CC_FAT), molecular function (MF_FAT), and KEGG pathways using *Homo sapiens* gene annotations and genome background within the DAVID Functional Annotation Tool (v6.7). Only terms with FDR < 0.05 and ≥ 2 genes were included.

Category	Term	Number of genes	Fold Enrichment	P-Value	FDR
GOTERM_BP_FAT	respiratory electron transport chain	12	9.7	3.40E-08	5.70E-05
	ATP synthesis coupled electron transport	11	10.1	9.80E-08	1.70E-04
	mitochondrial ATP synthesis coupled electron transport	11	10.1	9.80E-08	1.70E-04
	oxidation reduction	34	2.7	2.40E-07	4.00E-04
	cellular respiration	13	6.9	3.40E-07	5.90E-04
	electron transport chain	13	5.9	2.00E-06	3.40E-03
	oxidative phosphorylation	12	6.3	2.80E-06	4.80E-03
	cellular amino acid derivative metabolic process	15	4.7	4.00E-06	6.90E-03
	energy derivation by oxidation of organic compounds	13	4.7	2.20E-05	3.80E-02
GOTERM_CC_FAT	respiratory chain	13	9.3	1.20E-08	1.60E-05
	mitochondrial inner membrane	22	3.9	2.50E-07	3.40E-04
	mitochondrial membrane	25	3.4	3.10E-07	4.10E-04
	organelle inner membrane	22	3.6	8.40E-07	1.10E-03
	mitochondrial envelope	25	3.2	9.40E-07	1.30E-03
	mitochondrial respiratory chain	10	8.4	2.50E-06	3.40E-03
	mitochondrial membrane part	12	5.2	2.00E-05	2.70E-02
GOTERM_MF_FAT	heme-copper terminal oxidase activity	7	12.7	1.40E-05	2.10E-02
	oxidoreductase activity, acting on heme group of donors, oxygen as acceptor	7	12.7	1.40E-05	2.10E-02
	oxidoreductase activity, acting on heme group of donors	7	12.7	1.40E-05	2.10E-02
	cytochrome-c oxidase activity	7	12.7	1.40E-05	2.10E-02
KEGG_PATHWAY	Parkinson's disease	17	4.9	2.10E-07	2.40E-04
	Oxidative phosphorylation	17	4.9	2.60E-07	3.00E-04
	Complement and coagulation cascades	12	6.5	1.60E-06	1.90E-03
	Alzheimer's disease	17	3.9	5.50E-06	6.40E-03
	Huntington's disease	17	3.5	2.00E-05	2.30E-02

Table 4.1b, GO terms and KEGG pathways obtained using *Mus musculus* gene annotations and genome background within the DAVID Functional Annotation Tool (v6.7). Only terms with FDR < 0.05 and ≥ 2 genes were included.

Category	Term	Number of genes	Fold Enrichment	P-Value	FDR
GOTERM_BP_FAT	electron transport chain	13	6.9	3.90E-07	6.50E-04
	oxidation reduction	30	2.6	3.10E-06	5.10E-03
	regulation of body fluid levels	10	6.6	1.80E-05	3.10E-02
	blood coagulation	9	7.6	2.20E-05	3.70E-02
	coagulation	9	7.6	2.20E-05	3.70E-02
	hemostasis	9	7.5	2.50E-05	4.10E-02
GOTERM_CC_FAT	respiratory chain	13	12.9	2.60E-10	3.30E-07
	mitochondrial inner membrane	20	4.4	1.60E-07	2.00E-04
	mitochondrial membrane	22	3.9	2.40E-07	3.20E-04
	organelle inner membrane	20	4.1	3.50E-07	4.60E-04
	mitochondrial envelope	22	3.6	6.60E-07	8.60E-04
	organelle envelope	23	2.7	3.10E-05	4.00E-02
	envelope	23	2.7	3.20E-05	4.20E-02
GOTERM_MF_FAT	oxidoreductase activity, acting on heme group of donors, oxygen as acceptor	7	17.1	2.30E-06	3.20E-03
	cytochrome-c oxidase activity	7	17.1	2.30E-06	3.20E-03
	heme-copper terminal oxidase activity	7	17.1	2.30E-06	3.20E-03
	oxidoreductase activity, acting on heme group of donors	7	17.1	2.30E-06	3.20E-03
KEGG_PATHWAY	Oxidative phosphorylation	17	5.6	3.30E-08	3.70E-05
	Parkinson's disease	17	5.5	4.50E-08	5.20E-05
	Complement and coagulation cascades	12	6.9	8.80E-07	1.00E-03
	Alzheimer's disease	17	4	3.40E-06	3.90E-03
	Huntington's disease	17	4	3.70E-06	4.20E-03

Table 4.1c, GO terms and KEGG pathways obtained using *Danio rerio* gene annotations and genome background within the DAVID Functional Annotation Tool (v6.7). Only terms with FDR < 0.05 and ≥ 2 genes were included.

Category	Term	Number of genes	Fold Enrichment	P-Value	FDR
GOTERM_BP_FAT	electron transport chain	8	18.9	1.40E-07	1.90E-04
	generation of precursor metabolites and energy	12	7.1	7.10E-07	9.80E-04
GOTERM_CC_FAT	respiratory chain	7	32.1	3.70E-08	4.20E-05
	mitochondrial membrane	10	7.1	6.00E-06	6.70E-03
	mitochondrial inner membrane	9	8.3	7.30E-06	8.20E-03
	organelle inner membrane	9	8.1	9.10E-06	1.00E-02
	mitochondrial envelope	10	6.6	1.10E-05	1.30E-02
	organelle membrane	13	4.3	2.10E-05	2.40E-02
	mitochondrial part	10	5.8	3.40E-05	3.80E-02
KEGG_PATHWAY	Oxidative phosphorylation	14	7.4	1.80E-08	1.70E-05

Table 4.2. The most overrepresented biological functions ($n \geq 10$ molecules), the number of molecules from the RNA-seq dataset, enrichment p-value, and sample of genes and their direction of regulation in maternally-stressed embryos compared to embryos of control mothers. Genes were converted to Human Ensembl Gene IDs and analyzed via IPA (Ingenuity Systems).

Functions Annotation	# Molecules	p-Value	Molecules
quantity of cells	52	3.20E-03	↓ADD3, ↑AGR2, ↓AOC3, ↑ATF4, ↑BARHL2, ↓C3*, ↑C6, ↓CCND2, ↑CD200, ↑CISH, ↑CP, ↓CXCL12, ↑CYP2J2, ↓DNMT3B, ↑EEF1D, ↑...
quantity of blood cells	38	4.63E-04	↓ADD3, ↑AGR2, ↑ATF4, ↓C3*, ↑C6, ↓CCND2, ↑CD200, ↑CISH, ↓CXCL12, ↑EEF1D, ↑ETV6, ↑F2, ↑FUT4, ↑GNB3, ↑HBA1/HBA2*, ↑...
morphology of embryonic tissue	22	5.16E-03	↓AHCTF1, ↑DAD1, ↓DRAP1, ↑ETV6, ↑F2, ↑FGFR1, ↑HES5, ↓HIRA, ↓HIST1H1C, ↓HIST1H1D, ↓HIST1H1E, ↑KRT8*, ↓NCKAP1, ↓P...
development of sensory organ	20	5.37E-03	↑ATF4, ↑BFSP2, ↑CP, ↓DNMT3A, ↑FGFR1, ↓GJA8, ↑GNB3, ↑GPX4, ↑HES5, ↑KNG1, ↑LIM2, ↑NEUROD4, ↑POU3F4, ↑PSAP, ↓RARG, .
morphology of connective tissue	19	4.19E-03	↓ADD3, ↓DNAJA1, ↑ETV6, ↑F2, ↑HBA1/HBA2*, ↑HBB, ↑HBD, ↑HBZ, ↓HIST1H1C, ↓HIST1H1D, ↓HIST1H1E, ↑POU3F4, ↑PSAP, ↓R...
eye development	18	2.32E-03	↑ATF4, ↑BFSP2, ↑CP, ↓DNMT3A, ↓GJA8, ↑GNB3, ↑GPX4, ↑HES5, ↑KNG1, ↑LIM2, ↑NEUROD4, ↑PSAP, ↓RARG, ↑SOD1, ↑SOD2, ↑...
adhesion of tumor cell lines	15	8.89E-05	↑ALOX15B, ↑APOH, ↓B4GALNT2, ↓CXCL12, ↑CYP2J2, ↓DBF4, ↑F2, ↑KNG1, ↑NFKBIA, ↓PXN, ↓RAC1, ↓RAP1GAP, ↑S100P, ↑ST6G...
aggregation of cells	14	2.19E-03	↑CP, ↓CXCL12, ↑ETV6, ↑F2, ↑KNG1, ↑LGALS1, ↑NFASC, ↓NPHS1, ↓PLCB2, ↓PXN, ↑SERPINC1, ↑SERPIND1, ↓VASP, ↑VTN
adhesion of immune cells	14	6.60E-03	↓AOC3, ↑APOH, ↓C3*, ↑CD200, ↓CXCL12, ↑EZR, ↑F2, ↑FUT4, ↑KNG1, ↑NFKBIA, ↓RAC1, ↓RAP1GAP, ↓VASP, ↑VTN
morphology of eye	13	3.05E-03	↑ATF4, ↑BFSP2, ↑CP, ↓GJA8, ↑GNB3, ↑GPX4, ↑HES5, ↑NEUROD4, ↑PSAP, ↓RARG, ↑SOD1, ↑SOD2, ↑VSX1
abnormal morphology of extraembryonic tissue	12	5.29E-04	↑DAD1, ↑ETV6, ↑F2, ↑FGFR1, ↓HIRA, ↓HIST1H1C, ↓HIST1H1D, ↓HIST1H1E, ↓POU2F1, ↓PXN, ↑TFPI, ↓YAP1
kidney development	11	1.05E-02	↑EZR, ↑FABP2, ↑FGFR1, ↑HBB, ↑IGFBP2, ↑LCAT, ↓ODC1, ↑PSAP, ↑SOX4, ↑TFAP2B, ↑TKT
metabolism of amino acids	10	6.00E-04	↑ARG2, ↑ATF4, ↑BHMT, ↑BHMT2, ↑CKB, ↓CXCL12, ↑GPT, ↑HAL, ↓QDPR, ↑TDO2

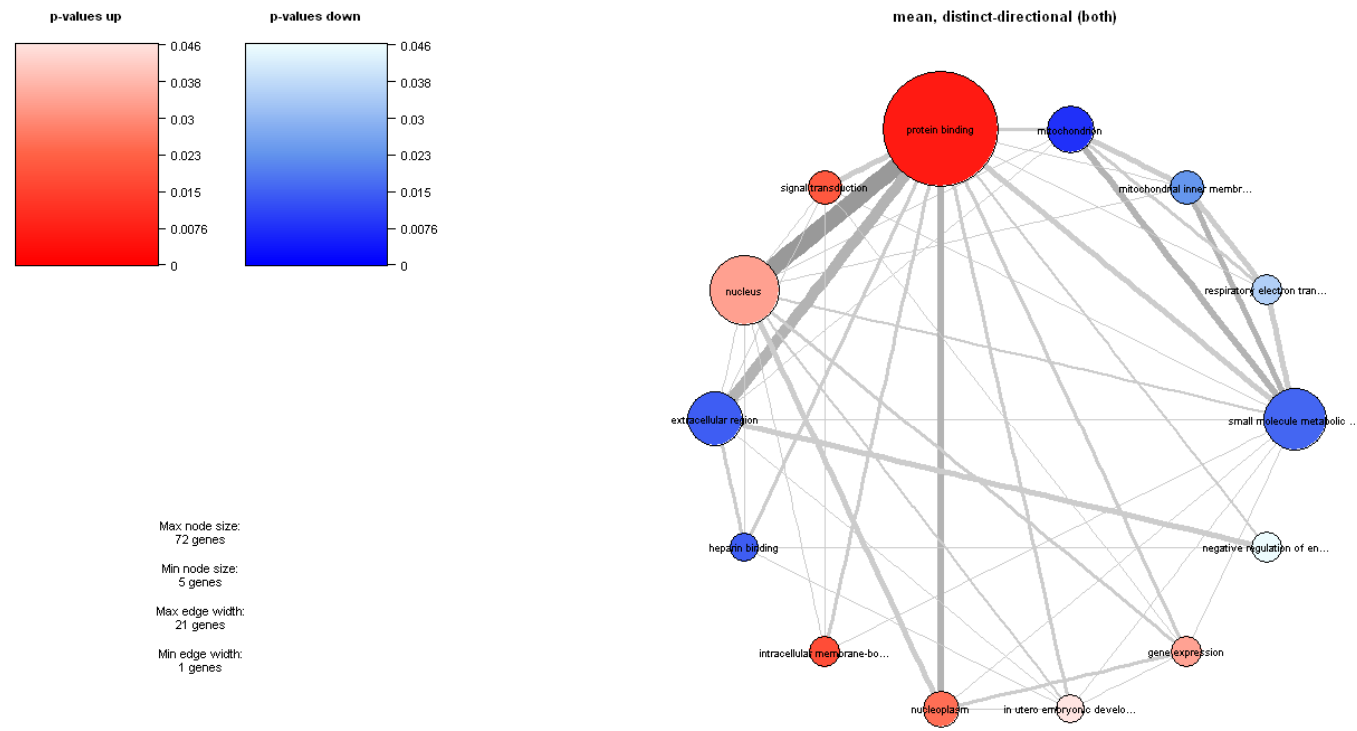


Figure 4.5, A network plot from PIANO gene set analysis (v1.04) of *Homo sapiens* orthologs of genes found to be differentially regulated in stickleback embryos as a result of maternal stress. Nodes represent sets of genes which are clustered together based on common functional annotations; their relative sizes denote the number of genes in the node. Edges connecting the nodes represent genes shared between nodes; their relative widths denote the number of genes shared. Red nodes contain genes that were up-regulated in stressed embryos compared to control and blue represents down-regulation in stressed compared to control. The intensity of a node's color represents the FDR-adjusted level of significance (all FDR are < 0.05)

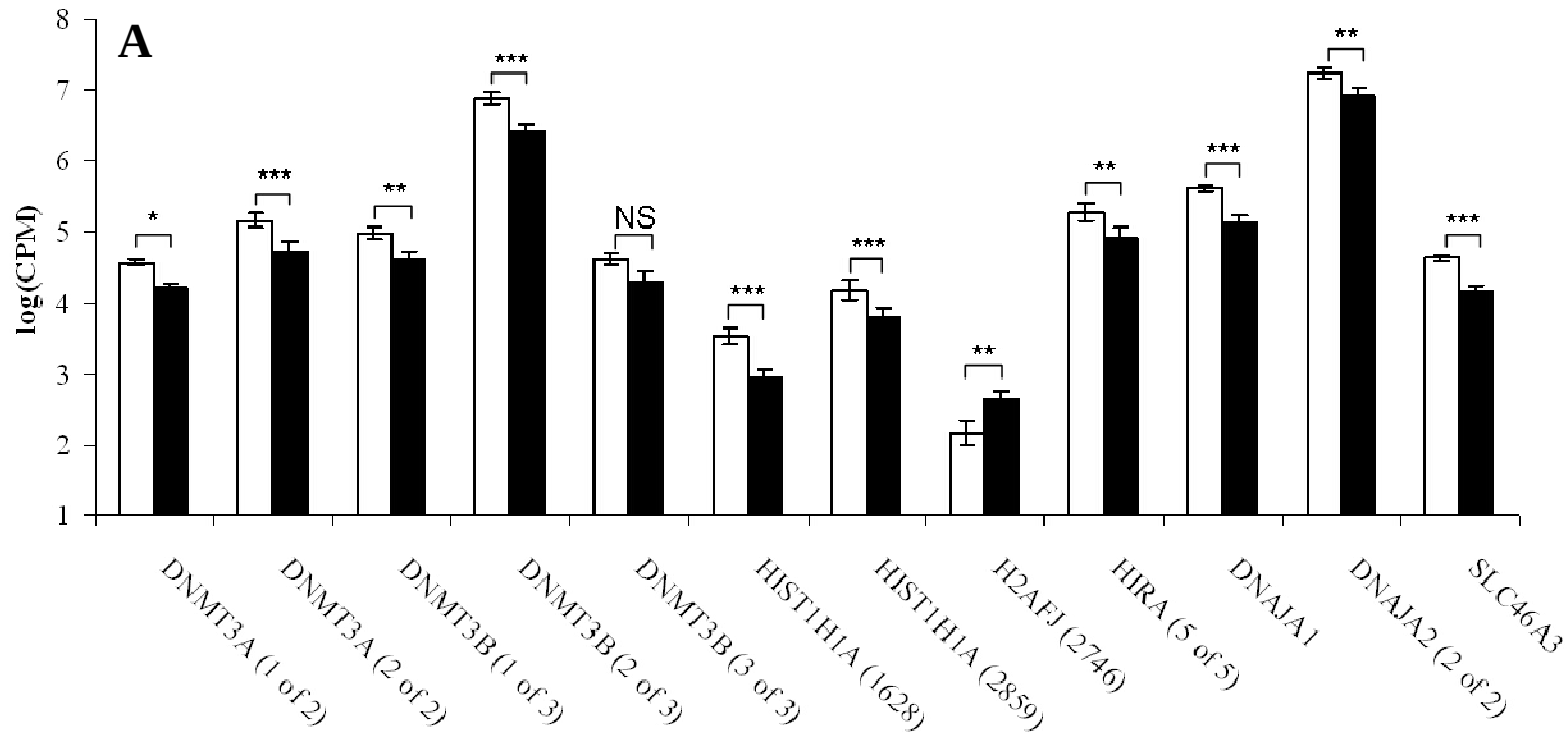


Figure 4.6, A) The expression of DNA methyltransferase genes, histone genes, and genes that interact with histone and DNA methylation in 3dpf stickleback embryos from mothers exposed to a predator and control mothers. B) The expression of genes involved in the proliferation and differentiation of neurons. Each bar represents the mean $\pm$ standard error of the logarithm of adjusted Counts Per Million mapped reads across clutches of embryos from $n=8$ mothers, and 10 pooled embryos per clutch. NS=raw p -value >0.05 , * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

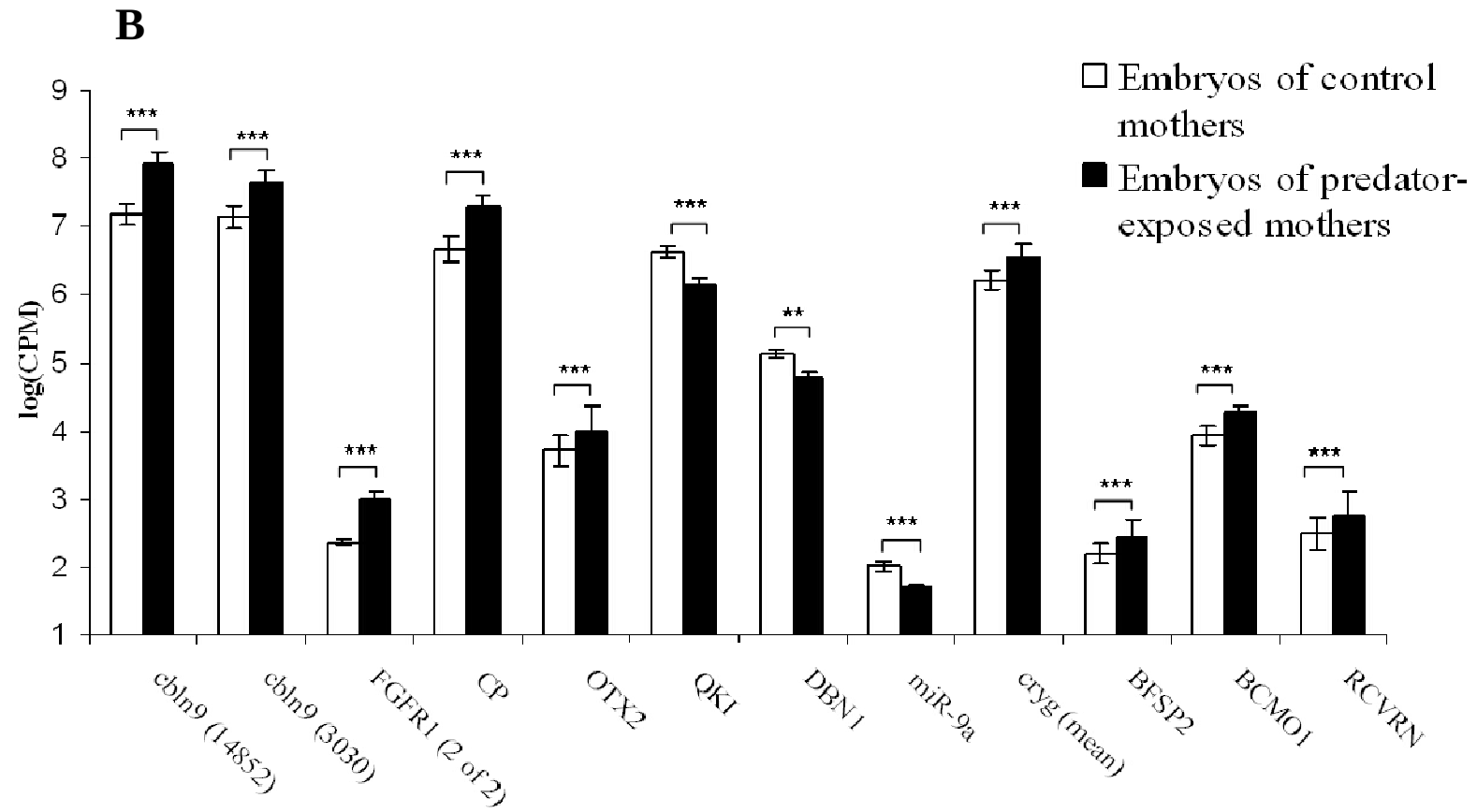


Figure 4.6 (continued)

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