Parental stress and its effects on the offspring of guppies (*Poecilia reticulata*) and zebrafish (*Danio rerio*)

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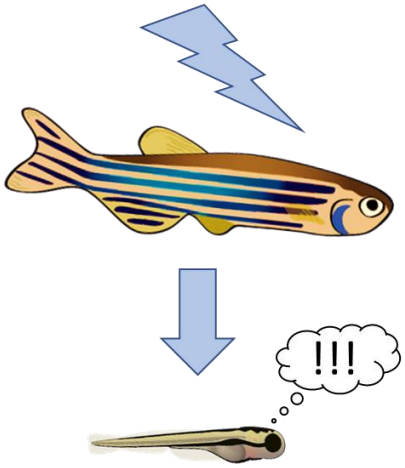

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Summary

Parental stress, either in the context of pregnancy or pre-conception, is increasingly recognised as a contributing factor to offspring disease risk. While the majority of data on the effects of parental stress derive from epidemiology and rodent models, small teleost fish are potentially useful models in which to study these processes. Following an introduction to the relevant concepts (Chapter 1), the subsequent experimental chapters concern two modes of parental stress and their phenotypic influences on the offspring in two different model teleosts: maternal gestational stress in guppies (Chapter 2), and paternal pre-conception stress in zebrafish (Chapter 3).

Chapter 2 focuses on the effects of maternal stress during the gestation period of guppies on the phenotypic characteristics (growth, behaviour, and stress responses) of the offspring. Mothers stressed during gestation produced offspring which grew more slowly during the early postnatal growth period and appeared to show a weakened stress response in adulthood. However, stress also shortened the mother's gestation period, and therefore subsequent phenotypic alterations in the offspring were likely resultant from shorter intrauterine development time.

Turning to the zebrafish, Chapter 3 Addresses the question of whether paternal stress has intergenerational effects on offspring phenotypes. The work revealed that behavioural and endocrine stress responses were ameliorated in the offspring of fathers exposed to chronic stress, concordant with similar findings from rodent models. It further demonstrated that an intergenerational effect on stress response can be transmitted following remarkably few exposures to a stressful stimulus in the paternal generation, suggesting that zebrafish are particularly sensitive to paternal intergenerational effects.

Following the discovery of paternal intergenerational effects of stress in zebrafish, Chapter 4 details the successful development of a method for isolating high-quality RNA from zebrafish spermatozoa. The chapter lays the groundwork for potential future experiments to investigate a hypothesised RNA-mediated mechanism of epigenetic inheritance – ideas which are expanded on in the general discussion (Chapter 5).

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Acknowledgements

This thesis is the culmination of not only four of the most challenging and most rewarding years of my life thus far, but of everything leading up to it. The first thing to be said, therefore, is that there are many people without whom, in one way or another, I would not have written this thesis. Sadly, there is not room to mention them all.

Thanks go first to my supervisors: Penelope Watt for her invaluable advice and support, for fostering a healthy habit of scrutiny, and for keeping me focused on doing a good job; Alireza Fazeli, for his infectious passion and warming encouragement, and for granting me an opportunity to spend some time at his lab in Tartu, Estonia (which was full of lovely people, and incidentally the place where I ended up actually finishing the thing); and Bill Holt, who, although more distantly associated with the project, could always be depended on for his insightful feedback. For the corrected version of the thesis, thanks are owed to the external and internal examiners, Julia Schroeder and Vincent Cunliffe, respectively, whose dedicated and critical evaluation of my work has enabled me to sculpt it into a far more robust and streamlined entity.

Much of the work presented would not have been possible without Helen Eachus, who was an informal mentor in the early stages and provided training in some of the methods which would become integral to the work. Neither would it have been possible without the care and attentiveness of the aquarium staff, Lynsey Gregory and Allison Blake, who also helped make the aquarium a friendly place to work. It was an honour to have been one of the last PhD students in the reign of Phil Young, who warmed the hearts of many like me who came and went during his 50-year tenure as head animal technician.

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Mere words are not sufficient to convey the gratitude I owe to my family. To my parents, Judy and Peter, without whose support, encouragement, and unconditional love I would not have been able to set foot on the scientific path. To my dear brothers Jack, Christopher, and John for putting up with me so well. To my late *bubbe* Ethel (who especially loved to discuss biology with me) and Grandpa Bill, both of whom would have been proud that this thesis could see the light of day. Thank you.

Declarations

With the exception of where stated, the work presented in this thesis is my own. The project was supervised by Penelope J. Watt (P.J.W.), Alireza Fazeli (A.F.), and William V. Holt (W.V.H.). The corrected version of this thesis includes several additions and alterations requested or suggested by the external and internal examiners, Julia Schroeder and Vincent Cunliffe (see Appendices 2 and 3).

Chapter 1: Introduction

The introductory chapter features components originally written for a comprehensive review on long-term effects of periconceptional stress which was published as a book chapter. A.F., W.V.H. (editors of the book), and P.J.W. provided feedback on the writing and are co-authors of the published review, a complete (open access) version of which can be found online via the following web link:

http://eprints.whiterose.ac.uk/122036/

Chapter 2: Gestational stress in guppies

Experiments were designed principally in consultation with P.J.W. and A.F. A summer student, Kelle Holmes, collected data on manual behavioural parameters of female guppies and their mature offspring. P.J.W. and W.V.H. provided initial feedback on the written chapter. A version of the chapter was submitted as a manuscript to *Journal of Experimental Biology* and subsequently the feedback of two anonymous reviewers was also implemented.

Chapter 3: Paternal stress in zebrafish

Experiments were designed principally in consultation with P.J.W. and A.F.; P.J.W. provided initial feedback on the written chapter.

Chapter 4: Zebrafish spermatozoal RNA islation

Paul Heath obtained bioanalyser images and W.V.H. provided initial feedback on the written chapter.

Appendix 1: Ionic stress in zebrafish embryos

This appendix comprises a small manuscript which was published as a ‘communication’ in the journal *Fishes*. Subsequently, feedback from two anonymous reviewers was implemented. The published (open access) article can be found online via the following web link:

https://www.mdpi.com/2410-3888/4/1/20

Image credit

The cartoon drawings of zebrafish and their larvae used on the title page and in Chapters 3, 4, and 5 were produced by Lizzy Grifiths and are freely available on her website (http://zebrafishart.blogspot.com/)

Chapter 1
General introduction

"The alternative to stress is death"

Ewan MacDonald, Honorary Professor of Public Health (University of Glasgow)

at a Sheffield meeting of stress researchers, February 2018.

In spite of its negative connotations, stress is an essential facet of animal physiology. Indeed, we would not exist as a species had our early hominin ancestors not felt appropriately stressed by the appearance of a predator. Although our neurophysiology has barely changed since the Palaeolithic, however, it must today contend with a legion of new pressures inherent to modern society in its unfathomable inter-connectedness. Consequently, the level of stress imposed by daily life does not always seem appropriate, and for an individual who suffers from anxiety or a related disorder, it is far from so. Such psychological illnesses are commonly associated with past experiences, and it has further become apparent that their roots can be laid down even earlier: in early development, or even in previous generations. That there exist biological mechanisms which have enabled our current state of mind to be influenced by events before we were born is a harrowing idea, and yet increasingly experimental and epidemiological evidence emerges to support it. This introduction will outline the key concepts of stress from a physiological perspective, the dysregulation of stress physiology in psychological illness, and evidence for the developmental and intergenerational underpinnings of this dysregulation. The case is then put forth for small teleost fish as models of parental effects of stress, specifically guppies (*Poecilia reticulata*) and zebrafish (*Danio rerio*) as models for gestational and paternal stress, respectively.

What is stress? Conceptual framework and physiological mechanisms

As aspects of the environment are in constant flux, organisms must continually respond to environmental changes through homeostatic mechanisms. Historically, definitions of the concept of stress have been difficult to pin down, but the different concepts will not be reviewed here, in favour of a clearer conceptual framework which is in line with a modern consensus. A broadly accepted definition is that stress entails a state of threatened homeostasis encountered following an excessive stimulus (a stressor), amelioration of which is beyond the reach of routine homeostasis mechanisms and instead necessitates stress responses to restore baseline function (Barton, 2006; Chrousos, 2009). Stress responses underly the body's often extraordinary ability to respond to unexpected danger, known as the *fight or flight* response (Sorrells *et al.*, 2009).

Adrenergic and HPA axis stress responses

The sensory stimuli that trigger the physiological stress response pathways are dependent on the evolutionary context of the organism. Initially, stress responses are activated following the coordinated engagement of neuronal circuits which are often specific to the environmental stimulus. For an in-depth review of stressor detection mechanisms, readers are directed to (Godoy *et al.*, 2018).

At the physiological level there are two key modes of stress response: the adrenergic stress response and the HPA axis stress response. The adrenergic response entails the rapid release of the neurotransmitters epinephrine (adrenaline) and norepeniphrene (converted from dopamine) from the adrenal gland situated above the kidneys (Wong *et al.*, 2012; Gordan, Gwathmey and Xie, 2015). These neurotransmitters engage with target neurons to initiate rapid *fight or flight* responses which are enacted over seconds, such as increases in heart rate (Gordan, Gwathmey and Xie, 2015), and rapid glucose production (Guest *et al.*, 2013). Via binding to corticotrophin-releasing hormone (CRH) neurons in the hypothalamus (Tilders *et al.*, 1985; Bugajski *et al.*, 1995; Bugajski, 1996), adrenaline can trigger a larger-scale stress response which entails the initiation and regulation of a suite of endocrine pathways embodied by the hypothalamic-pituitary-adrenal (HPA) axis (Smith and Vale, 2006; Bradley and Dinan, 2010) (Fig. 1). As the name would suggest, the principal structures of the HPA axis are the

hypothalamus (within the brain), pituitary gland (at the base of the brain) and adrenal gland. In short, activation of the HPA axis entails a cascade of neuronal and endocrine events, culminating in the release of glucocorticoid (GC) hormones as the primary stress response, which interact with glucocorticoid receptors (GRs) to enact a variety of secondary adaptive responses. In contrast to the adrenergic stress response, HPA axis responses are initiated over minutes (Herman *et al.*, 2016). For the framework of this project, the 'stress response' will primarily refer to this HPA axis stress response.

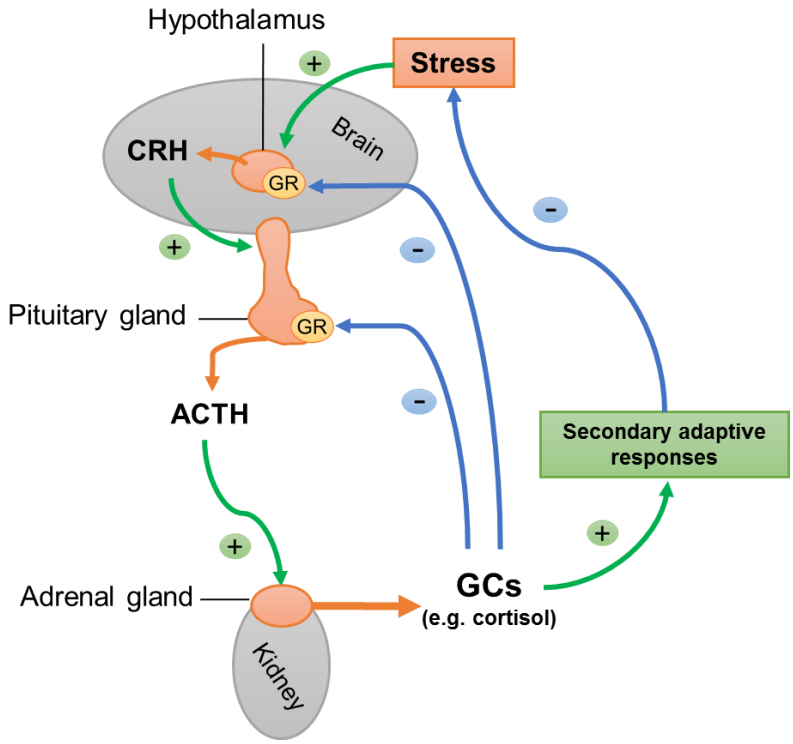

Fig. 1. Schematic diagram of the hypothalamic-pituitary-adrenal (HPA) axis. Upon registration of a stress stimulus by sensory neurones, information is relayed to the paraventricular nucleus (PVN) in the hypothalamus. The PVN continuously synthesises corticotropin-releasing factor (CRF) which, in response to sensory stimuli, is secreted into portal blood vessels which lead to the pituitary gland. Here, binding of CRF to its receptors induces the release of adreno-corticotropic hormone (ACTH), which enters the systemic circulation. Circulating ACTH reaches the adrenal cortex, situated along the perimeter of the adrenal gland, and upon reception stimulates the production and release of glucocorticoids (GCs) from the adrenal gland. GCs interact with glucocorticoid receptor (GR) to enact secondary adaptive responses, as well as the inhibition of the HPA axis via negative feedback. Green arrows / plus signs: stimulatory effects, red arrows / minus signs: inhibitory effects. Figure from (Ord, Fazeli and Watt, 2017).

The name ‘glucocorticoid’ derives from early observations that the hormones are involved in glucose metabolism (Kuo *et al.*, 2015). The primary GC hormone in humans is cortisol, which initiates and regulates a suite of adaptive responses: it interacts with the central nervous system to induce changes in cognition and awareness, inhibits costly immune functions (Lupien *et al.*, 2007), and stimulates hepatic glucose secretion (Jones, Tan and Bloom, 2012). Since the discovery of their immunosuppressive properties in the 1940s, GCs have provided useful anti-inflammatory drugs, which have been used to treat inflammatory diseases such as rheumatoid arthritis and asthma (Lupien *et al.*, 2007). To regulate the stress response, cortisol also has an inhibitory effect on HPA activity in the hypothalamus, which establishes a negative feedback loop essential to healthy HPA axis functioning (Herman *et al.*, 2016). The effects of cortisol and other GCs are mediated by the glucocorticoid receptor (GR), a cytosolic protein complex composed of heat shock proteins (HSPs) and expressed in almost every cell type in the body (Reichardt and Schütz, 1998). Following stress, GCs extensively occupy GRs, which enact transcriptional modifications either via binding with transcription factors, or as transcription factors themselves via direct interaction with glucocorticoid response elements (GREs) (Kuo *et al.*, 2015). Thus, cortisol induces up or down-regulation of several genes, leading to the synthesis of enzymes responsible for, e.g. neurotrophic factors and immunosuppressive factors. Cortisol dampens the stress response *via* the suppression of corticotrophin-releasing factor (CRF) and adreno-corticotropic hormone (ACTH) following GR binding in the hypothalamus and pituitary gland, respectively (Gjerstad, Lightman and Spiga, 2018) (Fig. 1).

Acute and chronic stress

A single encounter with a stressor, followed by the initiation of the stress response, amelioration of the stress and return to homeostasis describes the events of *acute stress*, for which the stress response apparatus is well-equipped. However, stress, by definition, is something we are not adapted to cope with excessively or repeatedly. Thus, excessive environmental stress impairs an organism’s fitness due to ‘wear and tear’ referred to as *allostatic load* (Schulte, 2014). The stress response is optimised towards restoring homeostasis in the face of single or *acute* stressors, while allostatic load accumulates in the face of prolonged or *chronic* stress, which may constitute several exposures, perhaps over a substantial

duration of an organism's life cycle (Sorrells *et al.*, 2009). In modern society, chronic stress often arises from a cocktail of negative socioeconomic factors such as job insecurity, financial problems, bereavement and interpersonal problems (Nargund, 2015). The harsher end of chronic stress may include a prolonged state of danger, such as domestic abuse, or severe resource detriment (*i.e.* famine).

Stress physiology is dysregulated in disease

Abnormal HPA axis functioning is associated with numerous pathologies, including both physical and psychiatric disorders. Both genetic and environmental factors may contribute to HPA axis dysfunction, which usually implicates imbalances of GCs, GRs, or both. Within the brain, GRs occur at high concentrations in the hippocampus, which is concerned with learning, memory, and attention (Lupien *et al.*, 2007), and in the limbic system, which is responsible for emotion (Harris *et al.*, 2013). Therefore, imbalances in levels of GCs or GRs have the potential to adversely affect attention span, emotional state, and other aspects of cognition. The association between GCs and psychiatric disorders first became evident in the 1950s through the increased incidence of psychosis in patients receiving GC therapy. These patients displayed gradually rising euphoria or dysphoria culminating in manic episodes, a condition which became known as "steroid psychosis" (Lupien *et al.*, 2007). Since then, imbalances in GCs and GRs have been implicated in major depressive disorder (MDD) (Alt *et al.*, 2010), schizophrenia (Bradley and Dinan, 2010), posttraumatic stress disorder (PTSD) (Palma-Gudiel et al. 2015) and almost all anxiety disorders (Faravelli *et al.*, 2012). The development of psychiatric disorders is frequently associated with chronic stress, such as childhood trauma, suggesting that HPA axis dysregulation may be induced by prolonged allostatic load at critical developmental stages (Heim and Nemeroff, 2001; Lupien *et al.*, 2009). Stress response physiology and its dysregulation in psychiatric illness therefore comprise a highly active area of research, in which animal models are an invaluable asset.

Psychological illness is frequently associated with physical ill health. This may owe partly to the fact that the HPA axis does not only regulate the response to stress, but also influences many other bodily processes including cardiovascular function, energy provision, fat deposition, and immune responses

(Kolber, Wieczorek and Muglia, 2009; Sorrells *et al.*, 2009; Bradley and Dinan, 2010). Thus, as well as affecting psychological health, disruption of HPA axis function through stress may have consequences for physical health. For example, excessive production of glucose resulting from overexposure to GCs may result in metabolic disorders such as type-2 diabetes (Bradley and Dinan, 2010). Furthermore, the immunosuppressive properties of GCs leave the body open to infection in states of chronic stress. In mice, for instance, chronic psychological stress and subsequent increase in endogenous GCs induces downregulation of antimicrobial peptides, increasing the severity of a bacterial skin infection (Aberg *et al.*, 2007). In contrast, however, in some cases of acute stress, GCs may also enhance the immune response in the central nervous system (Sorrells *et al.*, 2009).

Animal models in stress research

Since Erasistratus began to observe structural convolutions in the brains of stags and hares in the second century B.C. (Pearce, 2013), animal models have become integral to the field of neuroscience. The HPA axis comprises an ancient mechanism which is largely conserved across the vertebrate subphylum (De Marco *et al.*, 2013), and therefore methods for quantifying behavioural and physiological stress responses have been developed to study the HPA in rodents (Fig. 2). Typically, behavioural stress responses are characterised by anxiety-like behaviours. Several test paradigms, originally developed for rodents, aim to quantify these behaviours. For instance, the open field test relies on rodents' innate aversion to an unfamiliar environment, and generally uses the time spent preferentially at the edge of the test arena (thigmotaxis) as a measure of anxiety-like behaviour (Prut and Belzung, 2003). The light-dark preference test, meanwhile relies on rodents' aversion to bright light and uses time spent in darkness as a measure of anxiety-like behaviour (Bourin and Hascoët, 2003; Arrant, Schramm-Sapyta and Kuhn, 2013). In addition to behavioural phenotypes, physiological traits are employed as measures of stress response in rodents, such as serum glucose and corticosterone (the principal GC hormone in rodents) (Yao, Robinson, F. Zucchi, *et al.*, 2014).

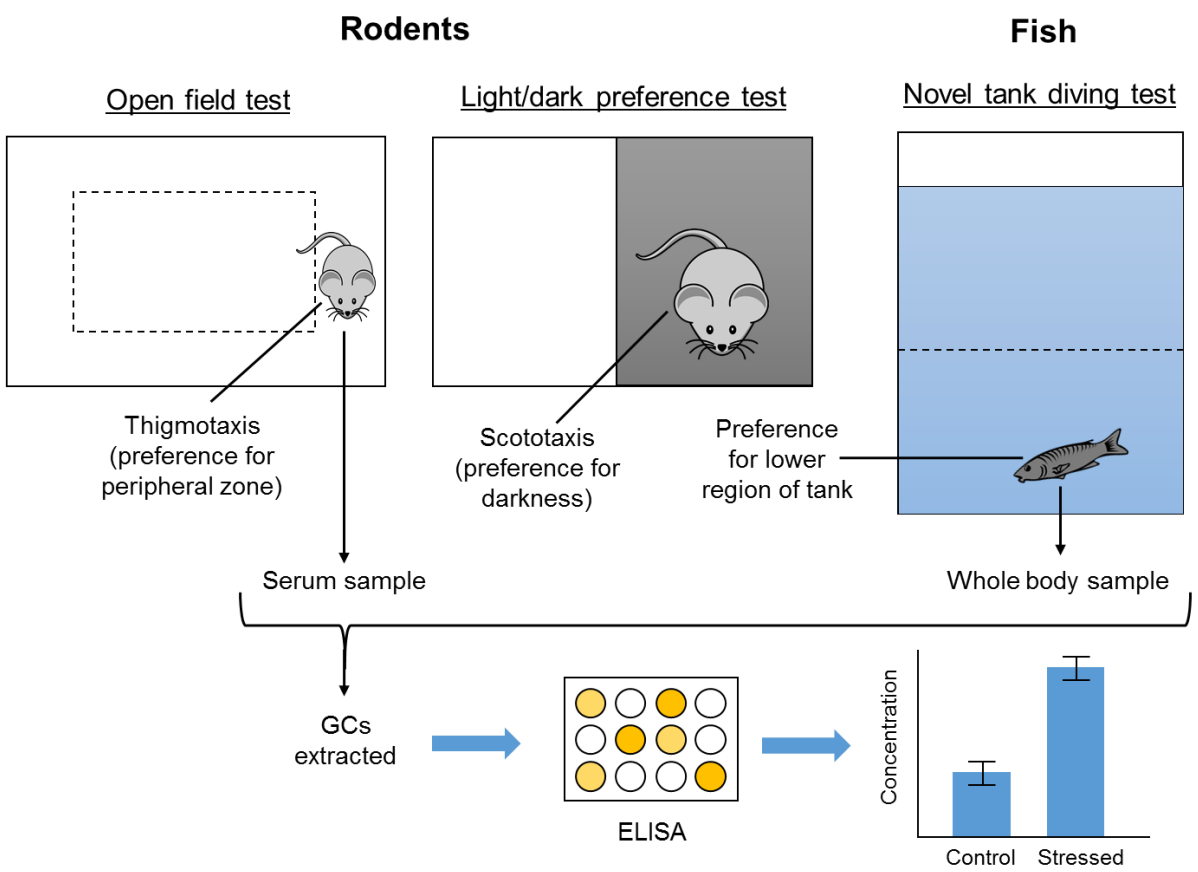

Fig. 2. Examples of quantitative behavioural and physiological stress phenotypes in rodents and fish. Strong anxiety-like behaviour (e.g., excessive thigmotaxis, scototaxis, or time spent in the lower region of a novel tank) is generally exhibited in response to HPA (mammals) or HPI (fish) axis activation by a stressor. Abnormal levels of anxiety-like behaviour detected using these measures may be indicative of dysregulation of the HPA or HPI axis, which may result from chronic stress. Following behavioural testing, cortisol may be extracted from serum or whole-body samples and quantified using ELISA. Figure from (Ord, Fazeli and Watt, 2017).

Rodents are among a wide range of animal models now applied in neuroscience, the choice of which always incurs a trade-off between the physiological similarity to humans and other factors such as cost, ease of maintenance, and ethical considerations. Increasingly, experimental neuroscientists are turning to small teleost fish, such as the zebrafish, as they are well-placed along this spectrum of practicality: they share several fundamental genetic, physiological, and anatomical similarities to mammals, yet possess several of the advantages also offered by invertebrate models, such as lower per-animal maintenance costs, rapid growth, and abundant reproduction. Teleost fish possess an equivalent to the HPA axis called the hypothalamic-interrenal (HPI) axis, within which the core stress response

mechanism is virtually identical to its mammalian counterpart (Clark, Boczek and Ekker, 2012). It is also noteworthy that fish, like humans, utilise cortisol as their principal GC hormone. Stimulation of the HPI axis typically corresponds with patterns of anxiety-like behaviour which appear homologous to their mammalian counterparts (Maximino *et al.*, 2010; Stewart *et al.*, 2012; Kalueff *et al.*, 2013). Thigmotaxis, for instance is a behaviour also observed not only in adult (Nema *et al.*, 2016) but also larval (5 days old) zebrafish (Schnörr *et al.*, 2011; Lundegaard *et al.*, 2015). This behavioural homology has enabled tests originally developed for rodents to be successfully adapted for use with teleost fish (Champagne *et al.*, 2010; Ariyomo, Carter and Watt, 2013), while unique assays to measure anxiety-like behaviour in fish have also been developed, such as the novel tank diving test, which uses the depth of a fish in an unfamiliar tank as a measure of anxiety-like behaviour (Egan *et al.*, 2009). The availability of these behavioural assays and the development of rapid, automated phenotyping protocols (Colwill and Creton, 2011; Pelkowski *et al.*, 2011; Stewart *et al.*, 2011; Nema *et al.*, 2016) have enabled the zebrafish to establish itself as a robust model in stress research.

Although the teleost stress response can be studied by direct pharmacological stimulation of the HPI axis (e.g. by use of stimulants such as caffeine; Schnörr et al. 2011), perhaps the most dramatic demonstration of the teleost stress response is observed following exposure to alarm substance (Fig. 3). Composed of chondroitin fragments released from the skin of conspecifics upon damage, alarm substance – named 'Schreckstoff' (scary stuff) by its discoverer, Karl von Frisch (v. Frisch, 1938) – is an olfactory cue which acts as a warning of predation (Mathuru *et al.*, 2012; Stensmyr and Maderspacher, 2012). Exposure to alarm substance stimulates cortisol production (Mathuru, 2016; Eachus *et al.*, 2017), and typical behavioural responses include freezing (inhibition of movement), erratic movement patterns (Speedie and Gerlai, 2008), and – if in groups – increased tendency to shoal (Nordell, 1998). Alarm substance therefore presents an effective model stressor which is nevertheless representative of natural conditions in which fish would encounter predators.

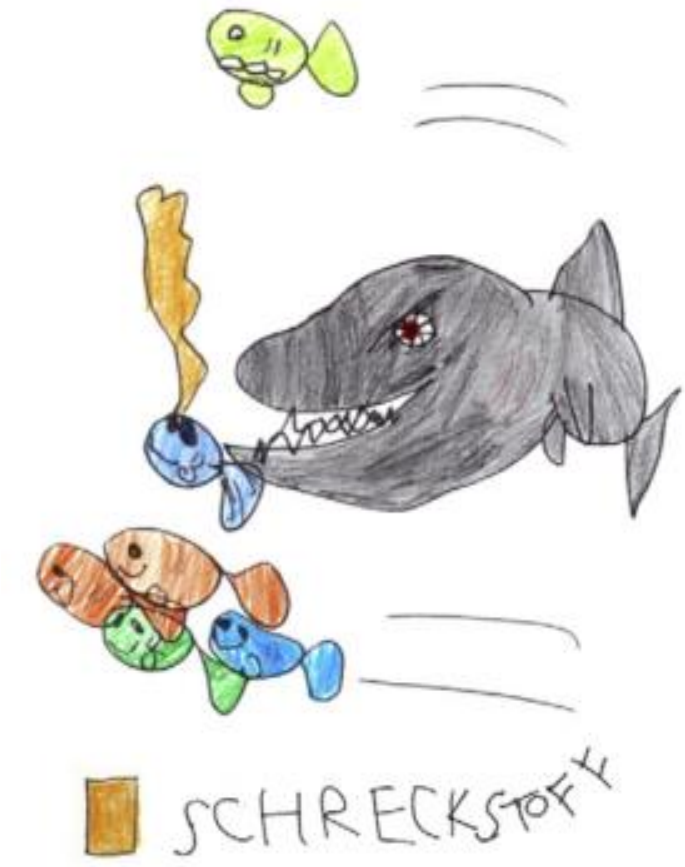

Fig. 3. Alarm substance or 'Schreckstoff' (scary stuff) is an endogenous chemical stressor released from the skin of certain teleost prey species upon mechanical damage and acts as a danger signal. Schematic taken from Stensmyr & Maderspacher (2012).

Epigenetic mechanisms and their role in HPA axis dysregulation

Long-term dysregulation of the HPA axis, and subsequently the development of psychiatric disorders, is frequently associated with chronic stress or stressful life events, especially during childhood (Heim and Nemeroff, 2001; Lupien *et al.*, 2009). Epigenetic mechanisms are now recognised as important in mediating long-term alterations to the stress response functioning in response to stress. Epigenetic mechanisms (*epi* meaning above) describe chemical modifications to DNA and chromatin which in some circumstances can facilitate 'fine-tuning' of gene expression in response to environmental cues (Feinberg, 2007; Van Soom *et al.*, 2014).

Defining epigenetics

The definition I will use for epigenetics is largely in line with the consensus definition established at the Cold Spring Harbour laboratory in 2008, at which an epigenetic trait was defined as "a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Berger *et al.*, 2009). In the context of this project (concerning environmentally-induced epigenetic effects), an epigenetic effect will be taken to refer to *an alteration in the phenotype resulting from alteration to chromatin factors governing gene expression*. An intergenerational epigenetic effect will refer to such an alteration which has been induced as a result of stimuli experienced in the previous generation. Epigenetic mechanisms comprise modifications to the chromatin environment, which extends from the DNA strand (cytosine modification) to the structural conformation of chromosomes (histone proteins), and also to associated non-coding RNAs. Phenotypic alterations resulting from heritable modification to any of these factors will be considered epigenetic effects.

A range of disparate alternative definitions of epigenetics are found in the literature. Conrad Waddington's original definition of epigenetics concerned "how genotypes give rise to different phenotypes during development" (Bird, 2007). However, this description would come to better describe the work of developmental biologists, who study how gene activity gives rise to specific phenotypes. An alternative definition given by Robin Holliday is "mechanisms of temporal and spatial control of gene activity during the development of complex organisms" (Holliday, 1990). This definition comes a step closer to the consensus in that it concerns the mechanisms underpinning the control of gene activity, but it is vague in that it does not elude to the specific nature of these mechanisms. A popular definition put forward by Arthur Riggs and colleagues was "the study of mitotically and / or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Bird, 2007). This definition adequately describes what epigenetics is *not* (mutational changes), but – as with Holliday's definition – leaves ambiguity as to the specific nature of the processes governing gene function. The Cold Spring Harbour definition overcomes this problem by encompassing to the notion that alterations occur within the chromosome, but above (*epi-*) the DNA sequence (*genetics*).

Specific epigenetic mechanisms

A broad and growing range of molecular processes can be considered as epigenetic mechanisms which are variously involved in fundamental developmental processes and in adaptive responses to environmental stimuli. The best known is the enzymatic methylation of the cytosine base of DNA into 5-methylcytosine which represses transcriptional activity by preventing the binding of transcription factors to promoter regions upstream of genes. DNA methyltransferase 1 (DNMT1) is among the key enzymes responsible for the establishment of methylation, and enables methylation patterns to be copied onto newly-synthesised DNA strands during cell division (Cantone and Fisher, 2013). DNA methylation is essential for fundamental processes in gametogenesis, embryogenesis, and development. It is essential for genomic imprinting – the process by which genes are silenced or expressed in a parent-of-origin-specific manner (Van Soom *et al.*, 2014). Levels of genomic methylation undergo dynamic changes throughout embryogenesis and embryonic development, in concert with changes in other epigenetic factors, but remain more stable at imprinted loci (He, Chen and Zhu, 2011). Aside from its role in development, DNA methylation is sensitive to environmentally-induced changes. For instance, toxicological studies have suggested that human DNA methylation can be altered by a variety of insults both during embryonic development and in adult life (Feil and Fraga, 2012). Furthermore, twin studies have revealed an enormous influence of the environment on the human methylome, suggestive of the role of methylation in facilitating phenotypic plasticity throughout the lifespan (Bell and Spector, 2012).

Another well-known class of epigenetic mechanisms comprises modifications to the histone proteins – those that provide structural framework for the chromosomes. Modifications to histone proteins by enzymes such as acetylates / deacetylases, methyltransferases, and phosphatases affect gene expression by altering the physical structure of chromatin in functionally important ways. For instance, acetylation of the histone tail makes the protein less compact, making the DNA strand is binds more exposed to transcription factors and thus its contents more likely to be expressed (Jenuwein and Allis, 2001). As with DNA methylation and its associated enzymes, the relative abundance of different histone modifications and levels of associated histone-modifying enzymes exhibit dynamic changes throughout embryogenesis and embryonic development, indicative of their role in these tightly-regulated processes

(Cantone and Fisher, 2013). Histone modifications are also environmentally responsive and have been found to be altered following exposure to toxicants (Feil and Fraga, 2012).

Another set of factors which, albeit does not comprise modification to the chemical or structural conformation of the genetic material may, still be considered epigenetic, are the noncoding RNAs. Comprising both long and small RNA species, noncoding RNAs are now believed to be essential regulators at the crossroads of genes, development, and environment. MicroRNAs (miRNAs) are small noncoding RNA molecules (~22 nucleotides) which modulate gene expression by either repressing translation or inducing degradation of target mRNAs (Hollins and Cairns, 2016). They are abundant in the brain and exhibit brain region-specific expression patterns in response to acute and chronic stress in animal models (Hollins and Cairns, 2016), suggesting they are important in neuroplasticity. Noncoding RNA is also thought to interact dynamically with DNA methylation in certain processes. For instance, piwi-interacting RNAs are required for the methylation-mediated silencing of transposable elements in mammals (He, Chen and Zhu, 2011). Only relatively recently has it transpired that RNA itself can be subject to covalent chemical modifications which give rise to an 'epitranscriptome'. Hitherto, very little is known about this 'new' level of organisation, but it is increasingly thought to play fundamental roles in development, environmental responses, and disease (Esteller and Pandolfi, 2017; Hsu, Shi and He, 2017).

Epigenetics and disease

Our understanding of phenotypic traits regulated by epigenetics stems from studies on animal models, and human diseases that have been linked to epigenetic alterations. For example, patients with Beckwith-Weidemann syndrome incur prenatal overgrowth, which is linked to loss of methylation of IGF2, among other chromatin alterations around growth-associated genes, indicating that epigenetic factors regulate tissue growth (Feinberg, 2007). Sufferers of Rett syndrome possess a mutation in a protein responsible for DNA methylation (MeCP2), and this is linked with erosion of neurodevelopment in childhood, suggesting a failure of the epigenetic mechanisms responsible for normal regulation of neurodevelopment (Feinberg, 2007).

There is also evidence for to link epigenetic changes with HPA axis dysregulation following chronic stress. For instance, poor maternal care in mice is linked with hypermethylation of genes associated with the stress response (e.g. glucocorticoid receptors) and with phenotypic outcomes which included increased plasma corticosterone levels and anxiety behaviour (Lee and Sawa, 2014). Another study showed that repeated psychological stress leads to increased phospho-acetylation of histone H3 in the hippocampus, but this is prevented by treatment with GR antagonists (Kolber, Wieczorek and Muglia, 2009). Furthermore, epigenetic dysregulation is also associated with psychological illness in humans: hypo-methylation of the *NR3C1* gene (encoding GR) is found in PTSD patients (Palma-Gudiel et al. 2015), and long term alterations in DNA methylation in *NR3C1* promoter regions has been suggested to mechanistically link MDD with childhood trauma (Alt *et al.*, 2010).

Periconceptional stress and long-term dysregulation

As previously mentioned, early life experiences can have a strong bearing on an individual's psychological health in later life, which may be due in a large part to neuroplasticity afforded by epigenetic mechanisms. A growing body of evidence, however, now suggests that long-term risk of psychiatric illness can be affected at even earlier stages: before birth, or even before conception (Painter, Roseboom and Bleker, 2005; Class *et al.*, 2013). Periconception refers to the period spanning from before conception to early pregnancy (Soom, Fazeli and Kuran, 2013). A wealth of evidence from epidemiology and animal models suggests that gestation is a critical period during which the environment can impact long-term development, while more recently evidence has emerged to implicate the paternal environment, before conception, in transgenerational effects of stress across the germ line (Anderson *et al.*, 2006; Gapp *et al.*, 2014). Although data presented across the vertebrate literature indicate that effects of the parental environment on offspring phenotype (maternal or paternal effects) can be induced by a number of mechanisms (see *Non-epigenetic mechanisms of parental effects*), there is increasing interest in the role of epigenetics which can help to explain the mechanisms of developmental programming at the most fundamental levels of biological organisation.

The notion that long-term disease risk is influenced by environmental conditions experienced in early developmental stages is encapsulated by the developmental origins of health and disease (DOHaD) hypothesis. The origins of DOHaD lie in historical observations from human populations by David Barker and colleagues who noted that birth weight is inversely correlated with later risk of heart disease, and subsequently proposed that prenatal nutrition was a contributing factor to adult disease (Grace, Kim and Rogers, 2011). Barker's hypothesis was that foetuses are receptive to environmental signals and can adaptively modulate aspects of development in preparation for a predicted set of postnatal environmental conditions. For instance, reception of lower levels of nutrients would prompt adaptive changes in fat and energy metabolism in preparation for a resource-poor environment (a derivation of Barker's hypothesis known as the 'thrifty phenotype'). These adaptive changes become maladaptive, i.e. manifest in disease phenotypes when the postnatal environment is mismatched with the prenatal one. This is illustrated by contrasting two human cohorts exposed to prenatal famine. Food shortages in caused by blockades in Holland at the end of WWII led to a period of famine known The Dutch hunger winter (Painter, Roseboom and Bleker, 2005; Roseboom, de Rooij and Painter, 2006). As the period of famine was relatively short (April 1944 – September 1945), individuals exposed to nutritional stress during early gestation were raised postnatally with good levels of nutrition (Schulz, 2010). The mis-matching of the two environments is purported to be responsible for the higher risk incurred by these individuals of metabolic syndromes in later life. By contrast, food shortages in the Soviet Union as a result of the siege of Leningrad were sustained over three years and individuals exposed in utero did not incur similar risk of metabolic syndrome (Stanner and Yudkin, 2001; Schulz, 2010).

It later emerged that not only maternal diet but exposure to other stressors, from smoking to trauma are associated with increased disease risk in the offspring consistent with DOHaD (Grace, Kim and Rogers, 2011). Some of the mechanisms underlying these associations have been characterised and comprise an interplay between maternal glucocorticoids and chromatin reorganisation. Examples of these mechanisms are reviewed in more detail in a later section (*Maternal / prenatal influences*).

A proposed extension to DOHaD would lead it to encapsulate environmental influences not only during embryonic development but also during gametic development in the previous generation. There is

evidence that environmental exposures such as smoking (Marczylo *et al.*, 2012) as well as obesity (Donkin *et al.*, 2016) can influence the ncRNA content of human sperm, and there is also some epidemiological evidence for a link between paternal obesity and breast cancer risk in female offspring (Romanus, Neven and Soubry, 2016). It has been suggested that paternal trauma or experience of violence, such as in the case of war veterans and holocaust survivors, may be paternally transmitted and influence offspring mental health (Vaage *et al.*, 2011). However, epidemiological evidence that paternal exposures are transmissible down the human germ line are still relatively limited (Yehuda, Halligan and Bierer, 2001; Vaage *et al.*, 2011), and such associations may be more likely to arise due to behavioural influences on children, rather than epigenetic transmission. However, recent evidence from model organisms has begun to shed light on mechanistic links between paternal pre-conception environment and offspring health.

An overview of periconception processes (gametogenesis and embryogenesis)

Gametogenesis describes the process by which haploid gametes (sperm and ova) are formed from early stage germ cells via successive stages of cell division (mitosis and meiosis) and differentiation (Finn, 2006).

In mammals, oogenesis begins from germ cells in the ovary. These undergo mitosis to produce oogonia which in turn undergo numerous mitotic divisions before entering the first stage of meiosis, giving rise to diploid primary oocytes. In mammals, the aforementioned stages occur before or shortly after birth. From here, meiosis is essentially 'paused' until a few hours prior to ovulation, many years later, when meiosis resumes to produce, successively, secondary oocytes followed by ova, at regular intervals. Only one ovum is produced from each cycle of meiosis, with additional chromatin from cell division disposed of in polar bodies (Finn, 2006; Rahman, Abdullah and Wan-Khadijah, 2008). Although in fish, oogenesis involves a similar succession from oogonia to ova, piscine spermatogenesis fundamentally differs from its mammalian counterpart in that the ovaries of fish possess germ cells at all stages of oogenesis (Lubzens *et al.*, 2010; Nakamura *et al.*, 2011). A constant supply of early stage germ cells is necessary to sustain high fecundity of teleost fish, most of which produce substantially more offspring

during their lifetime than mammals. For further details on piscine oogenesis, readers are directed to (Lubzens *et al.*, 2010) and (Nakamura *et al.*, 2011).

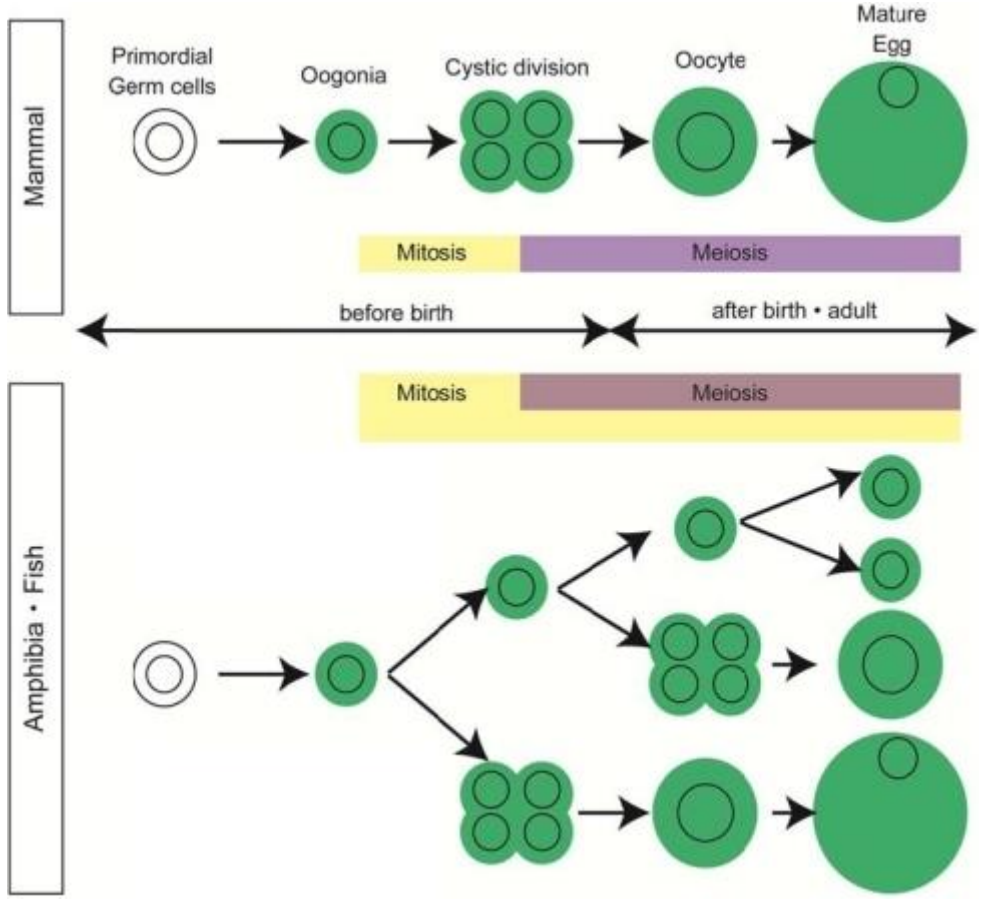

Fig 4. Overview of gametogenesis in vertebrates. In mammals (top) all mitotic stages are complete before birth and thus there is only a limited supply of oocytes, while in amphibians and fish (bottom) oogonia remain mitotic throughout life and thus a constant supply of oocytes is possible. Figure derived from Nakamura et al. (2011).

An important point to consider in the context of DOHaD is that exposure during gestation will not only expose the developing foetus, but also its germ cells. However, while in mammals, gestation is the only stage in which primordial germ cells could be perturbed, in fish these primordial cells could be exposed throughout the adult life of the mother, given the continuous nature of gametogenesis. Another important difference in relation to DOHaD is that because most fish are egg-laying and supply nutrients to the embryo via a yolk deposited in the ovum (Reznick, Callahan and Llauredo, 1996), alteration to the composition of this yolk may affect subsequent embryonic development.

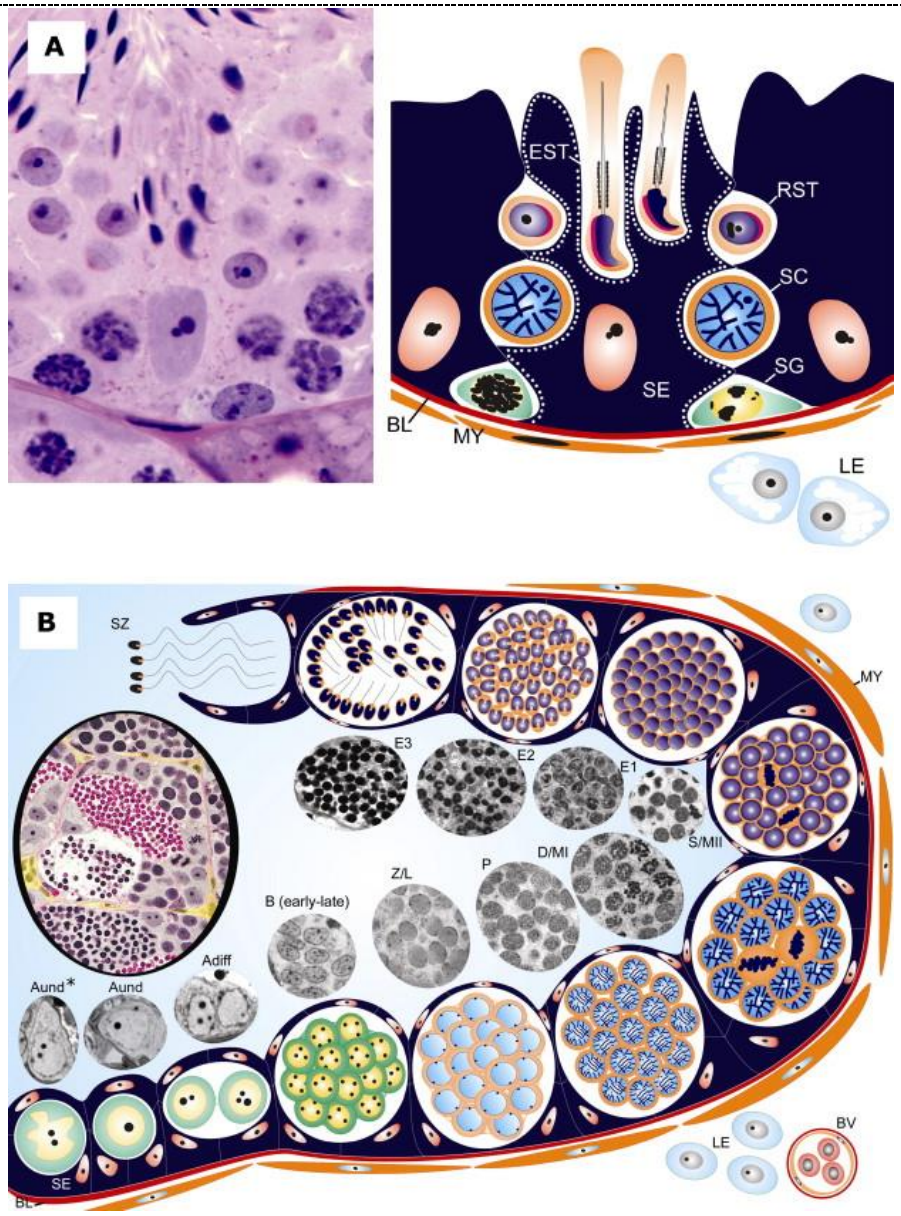

Fig. 5. Comparison between (A) non-cystic spermatogenesis in as is the case in mammals, reptiles and birds, and (B) cystic spermatogenesis as is the case in fish and amphibians. In each case, a cross-section is shown of the germinal epithelium in the testes where germ cell differentiation takes place. The latter is uniquely distinguished by featuring multiple cells at the same stage of differentiation enclosed within cysts formed from Sertoli cells (SE). For non-cystic spermatogenesis (A) the stages of germ cell maturation shown are spermatogonia (SG), spermatocyte (SC), round spermatid (RST) and elongated spermatid (EST). The cell completes its maturation in the epididymis (not shown) after leaving the seminiferous tubule. For cystic spermatogenesis (B), a continuous series of differentiation stages is shown from undifferentiated Type A spermatogonia (Aund) to spermatozoa (SZ). Figure derived from Schulz et al. (2010).

Spermatogenesis, like oogenesis, comprises a series of successive differentiations from germ cells to mature gametes. In mammals (as well as reptiles and birds), spermatogenesis occurs in the testis, within seminiferous tubules formed by Sertoli cells. Spermatogonia, the germ cells, undergo a set number of mitotic divisions, after which some daughter cells produce new spermatogonia while others undergo meiosis to produce spermatocytes, which mature into sperm (Finn, 2006). The Sertoli cells support all of these stages simultaneously (Fig. 5), but spermatozoa are not yet mature when they leave the seminiferous tubules, instead completing their maturation during passage through the efferent ducts and epididymis where they are stored prior to ejaculation (França, Avelar and Almeida, 2005). Key differences from mammalian oogenesis are that spermatogenesis begins at puberty and is continuous (i.e. mitosis ensures a constant supply of germ cells), and that each meiotic cycle results in four mature gametes instead of one. However, in mammals the Sertoli cells proliferate only until puberty (Schulz *et al.*, 2010).

Fish and amphibians possess a different mode of spermatogenesis called cystic spermatogenesis, whereby groups of Sertoli cells form cysts which enclose multiple differentiating germ cells, each deriving from a single spermatogonium (Leal *et al.*, 2009; Schulz *et al.*, 2010). This structural arrangement is thought to allow specific growth factors to be concentrated around cells in each developmental stage, resulting in a higher sperm yield. Unlike mammalian spermatogenesis, the Sertoli cells continue to proliferate throughout the lifespan of the animal. In addition, teleost fish testes do not possess an epididymis – the specialised compartment in which sperm matures in other tetrapods. Instead, sperm mature within the cysts which migrate towards the efferent ducts into which mature sperm are released, stored, and nourished prior to ejaculation (Uribe, Grier and Mejía-Roa, 2014; Boj, Chauvigné and Cerdà, 2015).

Vertebrate gametogenesis in both sexes is tightly regulated by a variety of hormones, including testosterone (Hansson *et al.*, 1976; Cabrera *et al.*, 2012). Consequently, endocrine disruption resulting from chronic stress could impact on gametogenesis. Mediated by GCs, stimulation of the HPA axis is now believed to have a direct inhibitory effect on the hypothalamic-adrenal-gonadal (HPG) axis, which drives key reproductive functions in both sexes, including spermatogenesis (Nargund, 2015).

Specifically, GCs inhibit the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, the downstream consequences of which include a reduction in testosterone, which is an essential regulator of spermatogenesis at several stages (Smith and Walker, 2014).

Each stage of female and male gametogenesis also entails extensive genomic reorganisation and chromatin remodelling. For instance, spermatogenesis sees chromatin undergo transitions of various histone variants, and during the final stages, in which chromatin becomes highly compacted, histones are replaced by specialised histone-like proteins known as protamines (Kimmins and Sassone-Corsi, 2005). Extensive epigenetic changes also take place during gametogenesis, which sees the erasure of biparental DNA methylation patterns and *de novo* remethylation in a sex-specific manner (Morgan *et al.*, 2005; Dean, 2014). Noncoding RNA is thought to be heavily involved in the regulation of gametogenesis, both in mammals (Smorag and Pantakani, 2017) and fish (Babiak, 2014).

The process of fertilisation, though complex and fascinating, will not be reviewed here. Readers are directed to (Ikawa *et al.*, 2010; Georgadaki *et al.*, 2016) for reviews on this topic.

After fertilisation, the male and female gametes do not immediately form an embryo which is ready to develop into an organism. Two haploid genomes must come together to form a single genome which must then become functional before the very first cell divisions and differentiations can occur. This process of early embryogenesis highly is complex and involves extensive chromatin remodelling and epigenetic changes. Paternal protamines, for instance are replaced by histones (Luense *et al.*, 2016), and there is another wave of epigenetic reprogramming in which the parental DNA methylation patterns are erased and a new methylome established (however, certain imprinted loci escape erasure) (He, Chen and Zhu, 2011; Feil and Fraga, 2012). This reprogramming occurs as part of the maternal-zygotic transition (MZT) whereby transcriptional control is transferred away from initial maternal factors, culminating in zygotic genome activation (ZGA) which ultimately facilitates the formation and subsequent development of the embryo (Meier *et al.*, 2018). Noncoding RNAs derived from the mother (Heyn *et al.*, 2014), and possibly even the father (Boerke, Dieleman and Gadella, 2007), are involved in this process, and there has been recent evidence for the involvement of RNA modifications (epitranscriptome) (Zhao *et al.*, 2017). A remarkable difference that has been discovered between

mammalian and zebrafish embryogenesis is that following erasure of gametic DNA methylation profiles, the newly-instated methylation profile is a mirror-image of the paternal methylation profile (Jiang *et al.*, 2013; Potok *et al.*, 2013). However, the extent to which this phenomenon is representative of teleost fish in general is not known.

In most mammals, embryos undergo a process of implantation whereby it adheres to the wall of the uterus, facilitating the formation of the placenta and providing the conditions for embryo nourishment and growth. This process does not occur in fish, though some viviparous (live-bearing) fishes have evolved placenta-like structures which facilitate active embryo nourishment during development (Banet, Au and Reznick, 2010). The subsequent stages of development will not be reviewed here, however readers are directed to Li (2002) for more information on embryonic development in relation to epigenetics and chromatin remodelling.

Given that epigenetic processes and chromatin remodelling during the processes of gametogenesis and embryogenesis ultimately lay the deepest foundations for the development of the new organism, there is considerable interest in the environmental control of these processes in relation to DOHaD (Soom, Fazeli and Kuran, 2013; Romanus, Neven and Soubry, 2016).

Maternal / prenatal influences

The prenatal period is now understood to be one of the most crucial stages of the human lifecycle in terms of our future health and wellbeing, both physically and psychologically. Prenatal stress, which may include domestic abuse, is associated with increased risk of adverse birth outcomes, such as preterm birth (Lilliecreutz et al., 2016), and growth retardation (Cottrell and Seckl, 2009), while evidence has grown over the past few decades to link psychological stress during gestation with longer term developmental outcomes. Depression during pregnancy, which affects up to 10% of women in the UK (Vigod and Wilson, 2016), with similar statistics reported in the US (Kinsella and Monk, 2009; Melville *et al.*, 2010; Stewart, 2011), has been shown to be a predictor of neurodevelopmental disorders in children and adolescents, while maternal stress during the first trimester of pregnancy is associated with increased risk of schizophrenia (Khashan *et al.*, 2008), suggesting neurodevelopment is sensitive

to stress during this early window. Prenatal famine exposure, studied in the Dutch famine cohort, has been associated with an increased risk of psychiatric disorders, including a two-fold increase in schizophrenia and related conditions (Brown *et al.*, 2000), while foetal alcohol exposure is associated with later onset of depression and anxiety (Hellemans *et al.*, 2010). Although several factors (e.g. postnatal influences) may play a role in these observed effects, there is extensive interest in, and growing evidence for, the impact of stress on prenatal development (particularly in relation to the HPA axis) *via* alterations to *in utero* physiology and epigenetic programming (Kinsella & Monk 2009; Palma-Gudiel et al. 2015). Such alterations involve complex interactions between the maternal environment, the placenta, and the developing embryo (Howerton *et al.*, 2013).

GCs play several essential roles in embryonic development, particularly of the neural tissues (Harris and Seckl, 2011), but overexposure to GCs resulting from stress has adverse consequences for prenatal development (Lupien *et al.*, 2009). In rats, chronic stress during pregnancy increases corticosterone in both mother and foetus (Takahashi, Turner and Kalin, 1998), which mediates increased anxiety-like phenotypes in adult offspring (Barbazanges *et al.*, 1996; Lupien *et al.*, 2009). GCs, which are employed for glucose production, are also increased in both mother and foetus during the state of chronic stress induced by under-nutrition (Blondeau *et al.*, 2001), and as a result of alcohol exposure (Liang *et al.*, 2011). Thus, HPA axis dysregulation resulting from overexposure to GCs may underlie pathologies associated with maternal stress and undernutrition (Brown *et al.*, 2000), as well as foetal alcohol syndrome (Hellemans *et al.*, 2010).

The molecular aetiology of developmental programming of the HPA axis in response to prenatal stress is likely to include epigenetic alterations to target chromatin, as chromatin organisation affects the levels of expression of associated gene sequences (Cottrell and Seckl, 2009). Differential expression of three key placental genes have been implicated in prenatal stress: 11β-hydroxysteroid dehydrogenase type 2 (*11β-HSD2*), glucocorticoid receptor (*NR3C1*) (Conradt *et al.*, 2013), and O-linked N-acetylglucosamine transferase (*OGT*) (Howerton *et al.*, 2013). In addition, a host of regulatory RNAs have been implicated in developmental programming. However, these are likely only a few of the

factors contributing to the byzantine dialect between the environment, placenta, and developing embryo, in which much remains to be elucidated.

11β-HSD2 regulates foetal GC levels by converting cortisol into inert cortisone, thus protecting the foetus from GC overexposure. Maternal stress, anxiety, and under-nutrition induce down-regulation of *11β-HSD2*, which has been shown to correlate with reduced birth weight, as well as HPA axis dysregulation and anxiety-like behaviour (Cottrell and Seckl, 2009; Conradt *et al.*, 2013). Similar outcomes are observed in homozygous knockout mice (*11β-HSD2*-/-) (Cottrell and Seckl, 2009). In rats exposed to chronic prenatal stress, foetuses possess reduced expression of *11β-HSD2*, and increased CpG methylation in the *11β-HSD* promoter region in hypothalamic tissue (Peña, Monk and Champagne, 2012), while human mothers who report anxiety during pregnancy possess greater placental methylation of *11β-HSD2* (Conradt *et al.*, 2013). Collectively, the evidence suggests that *11β-HSD2* is an important component of the molecular interface between the maternal environment and the developing foetus, and thus significant to the aetiology of stress-induced developmental pathologies.

NR3C1 is the gene encoding the glucocorticoid receptor (Conradt *et al.*, 2013). Like GC, GRs are essential for normal development. For example, homozygous GR knockout mice die in the first few hours of life due to severely impaired lung development (Kolber, Wieczorek and Muglia, 2009). Likewise, reduction in *NR3C1* expression by 30-50% in transgenic mice leads to exaggerated HPA axis responses to stress (Michailidou *et al.*, 2008). There is now evidence to link this differential expression to targeted epigenetic reprogramming in response to prenatal stress. For example, mothers who report depression during pregnancy have higher methylation of placental *NR3C1* (Conradt *et al.*, 2013), while domestic abuse during pregnancy is significantly associated with methylation in the *NR3C1* promoter in adolescent offspring (Radtke *et al.*, 2011). A recent meta-analysis of human DNA methylation data from 977 individuals revealed that methylation of a single CpG site in the promoter region of *NR3C1* was significantly associated with prenatal stress (Palma-Gudiel et al. 2015).

Another factor recently implicated in the link between prenatal stress and disease risk is O-linked N-acetylglucosamine (O-GlcNAc) transferase (Ogt). The enzyme is a key cellular regulator which

modifies, by addition of O-GlcNAc, protein targets responsible for chromatin remodelling (e.g. RNA polymerases, histone deacetylases) (Howerton *et al.*, 2013). Ogt also preferentially associates with TET proteins (regulators of DNA methylation state) in close proximity to CpG-rich transcription start sites (Vella *et al.*, 2013). Maternal stress leads to reduced expression of placental *OGT*, and *OGT*-knockout mice develop HPA axis dysregulation characteristic of that induced by stress in early pregnancy (Howerton and Bale, 2014). Deficiency of Ogt is hypothesised to underlie observations of male-biased risk of neurodevelopmental disorders, as it escapes X chromosome inactivation in the placenta and is thus expressed at higher levels in females (Howerton *et al.*, 2013). Furthermore, because O-GlcNAc is produced from glucose, Ogt is a potent sensor of cellular nutritional status, and is thought to be similarly responsive to other aspects of the environment (Zachara and Hart, 2004; Love and Hanover, 2005; Vella *et al.*, 2013). Because of this, and because of its interaction with TET proteins and other factors associated with chromatin remodelling (Howerton *et al.*, 2013; Vella *et al.*, 2013), it is plausible that Ogt is a key mediator of stress-induced epigenetic alterations associated with *11β-HSD* and *NR3C1*.

Given their abundance in the brain and responsiveness to acute and chronic stress, there is now evidence that miRNAs are key mediators of stress-induced neurodevelopmental pathologies. In response to gestational stress, one study revealed that the brains of new-born mice exhibit differential expression of over 336 miRNAs (Zucchi *et al.*, 2013). Several of these miRNAs are involved in neurodevelopment and have been implicated in psychiatric disorders, including miR-219, which is up-regulated in patients with schizophrenia. This differential miRNA expression was subsequently demonstrated to persist into the F2 generation, suggesting miRNAs may play a role in transgenerational programming of the oocyte (Yao, Robinson, F. C. Zucchi, *et al.*, 2014), and thus may mediate epigenetic inheritance of disease risk. Interestingly, among the down-regulated miRNAs were miR-200b, which is implicated in uterine contractibility, and thus may provide a putative mechanistic explanation for preterm birth associated with gestational stress (Yao, Robinson, F. C. Zucchi, *et al.*, 2014).

When considering long term implications of prenatal stress on HPA axis development, the neuroplasticity of the early postnatal brain (Cramer *et al.*, 2011) must also be considered, as some lines

of evidence suggest that alterations to HPA axis development in the prenatal period can be attenuated by intervention in the neonatal period. For example, rats exposed to handling during the preweaning period exhibit permanent reductions in corticosterone secretion and GR expression (Welberg and Seckl, 2008), and consequently, neonatal handling has been found to eliminate some of the adverse effects of foetal alcohol exposure, such as increased weight gain (Weinberg, Kim and Yu, 1995), and HPA axis hyperactivity (Ogilvie and Rivier, 1997). However, subsequent experiments have produced conflicting results in this regard (Gabriel *et al.*, 2000).

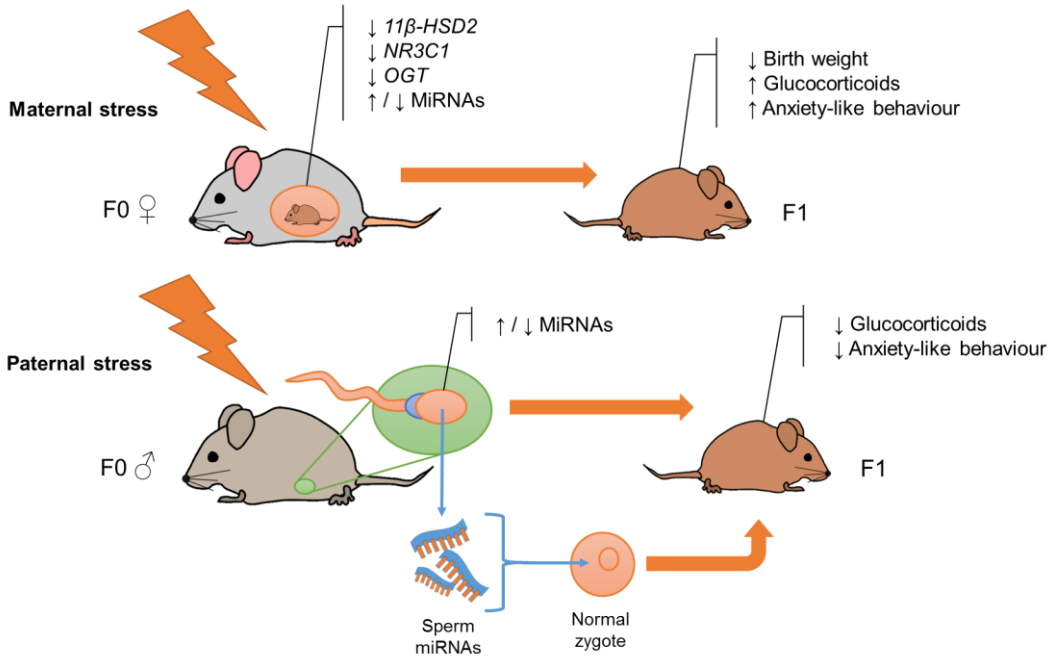

Fig. 6. Summary of molecular pathways altered by chronic stress in the periconception period, and phenotypic effects in offspring. Chronic maternal stress in the prenatal period induces down-regulation of 11β-HSD2 (Conradt et al. 2013), NR3C1 (Cottrell and Seckl 2009; Conradt et al. 2013), and OGT (Howerton and Bale 2014) in the placenta and / or foetus, and postnatal phenotypes indicative of HPA axis hyperactivity (increased glucocorticoids and anxiety-like behaviour) (Lupien et al. 2009). Chronic paternal stress in early life (Gapp et al. 2014), adolescence, or adulthood (Rodgers et al. 2013) alters sperm RNA composition and induces phenotypes indicative of suppressed HPA axis activity (decreased glucocorticoids and anxiety-like behaviour) in subsequent offspring. Insertion of sperm RNAs from stressed males into normal zygotes by microinjection recapitulates the paternal stress phenotypes in resultant pups (Gapp et al. 2014; Rodgers et al. 2015). Figure from (Ord, Fazeli and Watt, 2017).

Paternal influences

The majority of literature on parental environmental influences on HPA axis development has focused on maternally-mediated effects. Understandably, given that humans are confined to the maternal environment for the first nine months, it was long thought that the paternal (father's) environment was of little importance. However, it has since become apparent that the spermatozoon provides to the embryo more than simply a haploid genome, and subsequently the paternal environment (particularly paternal stress) is becoming increasingly implicated in HPA axis dysregulation. A handful of studies have identified heritable alterations in measurable phenotypic aspects of HPA axis activity induced by paternal stress at different developmental stages. For instance, male mice subjected to a chronic stress paradigm (maternal separation) in early life transmit depression-like symptoms and dampened stress responsivity to their offspring (Gapp *et al.*, 2014), while chronic stress during either adolescence or spermatogenesis (approx. 42 days in mice) results in dampened corticosterone production in the offspring (Rodgers *et al.*, 2013). Similarly, paternal fasting during spermatogenesis alters serum glucose in murine offspring (Anderson *et al.*, 2006). Spermatogenesis, therefore, appears to be a critical window during which environmental exposures can leave a mark on subsequent offspring.

In contrast to maternal effects, in which germ line-mediated effects are difficult to discern from in-utero effects on development, paternal effects on phenotype are more likely to represent the germ line transmission of environmental information. Germ line epigenetic inheritance has long been a puzzle due to the problem of erasure: the DNA methylation status of the parental genomes are re-set during the first few cell divisions, and thus it is widely thought that most alterations to methylation acquired during the parents' lifetime are erased (Cantone and Fisher, 2013). Although acquired methylation changes may escape erasure in some cases, many experimental studies of paternal epigenetic inheritance have failed to associate inherited phenotypes with alterations to paternal sperm methylation (Feil and Fraga, 2012), and thus it has become clear that DNA methylation is insufficient to explain paternal epigenetic inheritance. Subsequently, attention turned to other epigenetic mechanisms and their potential to escape erasure, specifically the histone-like protamines which help to package genetic

material in the sperm nucleus, and noncoding RNA which can be delivered in sperm on top of the primary genomic consignment.

Stress may influence offspring phenotypes via post-translational modification to sperm chromatin structure, specifically histones and protamines. Chromatin undergoes extensive reorganisation during spermatogenesis, in which most histones are supplanted by protamines (Luense *et al.*, 2016). Numerous unique protamine modifications, particularly acetylation and methylation, have been discovered in human and mouse sperm (Brunner, Nanni and Mansuy, 2014), prompting the hypothesis that these decorations may play an important role in the epigenetic regulation of embryonic development following fertilisation, and furthermore may represent mediators of germline epigenetic inheritance (Luense *et al.*, 2016). Although most paternal histones and protamines are believed to be replaced by maternally-inherited histones soon after fertilisation (Cantone and Fisher, 2013), sperm histone marks retained at fertilisation have recently been reported to be essential for correct gene expression in *Xenopus* embryos (Teperek *et al.*, 2016). There is still very little known regarding sperm histone and protamine post-translational modifications, including the extent to which they may be subject to external environmental influences, and thus more attention is needed in this area of research.

In contrast to the stable transmission of altered DNA methylation or protamine marks in sperm, for which there is only limited evidence, a role of spermatozoal RNA in paternal epigenetic inheritance has been clearly demonstrated. In their studies of inheritance of paternal stress phenotypes, Rodgers et al. (2013) and Gapp et al. (2014) both found that stressed males exhibited differential expression of miRNA in sperm. Gapp et al. (2014) also showed that injection of spermatozoal RNA from stressed males into normal zygotes recapitulated paternal stress phenotypes in the offspring. Concordantly, in a follow-up to their 2013 study, Rodgers et al. (2015) demonstrated that injection of just nine spermatozoal miRNAs found to be upregulated under stress into normal zygotes recapitulated the paternal stress phenotype detected in their previous study. Interestingly, both long and short noncoding RNAs have since been demonstrated to play different roles in paternal inheritance of stress phenotypes, but injection of long or short fractions induce different phenotypic alterations (Gapp *et al.*, 2018).

Comparing the published experiments provides further clues as to the underlying mechanism of germ cell alteration. HPA axis dysregulation in offspring occurs in response to paternal stress in early life (Gapp *et al.*, 2014), adolescence (Rodgers *et al.*, 2013), and adulthood (Rodgers *et al.*, 2013; Dias and Ressler, 2014), and the same phenotype has been recapitulated by the injection into normal zygotes of both total RNA from the sperm of traumatised males (Gapp *et al.*, 2014), and of specific miRNAs associated with paternal stress (Rodgers *et al.*, 2015). This suggests that even though the phenotypes were induced in response to stress in different developmental stages, the underlying mechanism may be very similar if not the same. ncRNAs may be transported to maturing sperm *via* microvesicles, and likely interact with the zygotic transcriptome to influence the outcome of genomic reprogramming towards the observed phenotypes. It is also possible that stress-induced testosterone deficiency may play a role in miRNA-mediated inheritance, as testosterone is known to regulate the expression of several miRNAs in Sertoli cells (Panneerdoss *et al.*, 2012; Smith and Walker, 2014). Interestingly, two of the nine stress-responsive sperm miRNAs discovered by Rodgers et al (2013) – miR-25c and miR-375 – are also regulated by testosterone, as shown using a mouse model of testosterone deprivation (Panneerdoss *et al.*, 2012). MiR-375 is relatively well-characterised in terms of function and is important for the development of the pancreas and pituitary gland, while little is known about the miR-25 family except that they are implicated in cardiac function.

Although the underlying mechanisms remain elusive, it is clear that environmentally-induced reprogramming occurs not just in the developing embryo, but in developing germ cells. The observation that the same phenotypes induced by paternal stress in early life and adolescence is also induced by stress during spermatogenesis suggests that, rather than resulting from long-term alterations to germ cell precursors, modifications to maturing germ cells occur transiently in response to long term alterations to HPA axis functionality. If this is the case, reversal of pathologies by effective treatment may halt or at least reduce the modification of maturing germ cells, preventing the inheritance of pathologies. This would mean that the potential for such prevention would depend directly on the availability of effective treatment for said pathologies.

Non-epigenetic mechanisms of parental effects

The demonstrated importance of epigenetic mechanisms in transmitting influences of the parental environment notwithstanding, it is important to acknowledge that several other mechanisms have been documented to contribute to 'parental effects' (as they are referred to in the evolutionary literature). A maternal (or paternal) effect is defined as an influence of the parental fitness phenotype on the offspring phenotype. Offspring fitness is influenced by the parental genotype and the parental environment (maternal / paternal environmental effects) (Rossiter, 1996). The following section outlines some of the various mechanisms of maternal and paternal environmental effects on the offspring phenotype reported in the insect, avian, and fish literature. These include nutrient provisioning, transmission of cytoplasmic factors (e.g. hormones), and influences of parental behaviour on both pre- and postnatal offspring (e.g. learning).

The provision of nutrients to embryos, usually *via* the yolk, is an important determinant of offspring fitness. The level of nutrition allocated to the yolk limits the rate of growth in several species, and yolk nutritional content is in turn limited by the nutritional status of the mother (Rossiter, 1996; Bonduriansky and Day, 2009). In zebrafinches, poor neonatal diet constrains the rate of reproduction in adulthood, likely due to a constraint on the number of lipoproteins (cells responsible for uptake of lipophilic antioxidants) that can be established in early development, limiting the uptake of antioxidants needed for reproduction (Blount *et al.*, 2006). However, mothers can alter nutrient allocation to the yolk, facilitating adaptive responses. In guppies, mothers experiencing low nutrition produce fewer but heavier offspring, attributable to increased fat reserves allocated to the yolk in response to a resource poor environment (Reznick and Yang, 1993). In species with parental care, nutrients fed to offspring by either parent can affect early growth and development. In insects, the paternal diet can enhance offspring fitness *via* the mother by supplying a nuptial gift (Vahed, 1998).

Transmission of hormones to oocytes is already known to play a role in maternal control of diapause in insects. In most cases, mothers that experience short days produce a higher proportion of diapausing eggs than those experiencing longer days, and the inclusion of diapause hormone (DH) in eggs in

response to photoperiod or temperature facilitates this (Hockham, Graves and Ritchie, 2001). However, in some species the maternal influence manifests later in postnatal development; the authors conceded that the mechanism for this delay remained elusive at the time of publication. In birds, mothers can regulate the androgen content of eggs, which affect growth rates, sex determination, and behaviour of offspring (Verboven *et al.*, 2003; Groothuis *et al.*, 2005; Niall Daisley *et al.*, 2005). Steroid hormone levels in the mother can strongly fluctuate as a function of the environment, particularly the social environment (e.g. aggressive behaviour) (Groothuis *et al.*, 2005). Factors beneficial to immune function can also be transmitted to offspring including antibodies and carotenoid compounds (Blount *et al.*, 2002). Owing to this protection, more resources can be devoted to growth, leading to an overall fitness benefit. In some moth species, nuptial gifts given by the father to the mother can be endowed with defensive compounds, which can be incorporated into eggs (Dussourd *et al.*, 1989). An example of a more direct paternal influence occurs in blennies, the males of which may transfer antimicrobial substances to eggs *via* specialised anal glands (Giacomello, Marchini and Rasotto, 2006).

Maternal and paternal effects can also occur as a result of parental behaviour either before or after the birth of the offspring. Environmental conditions can affect the mother's choice of egg laying site, which is critical as it determines the first external environment encountered by the offspring (Rossiter, 1996). In species with extended parental care, parents can transmit behavioural traits to offspring non-genetically *via* learning. For example, many birds learn to sing by listening to their parents' songs (Freeberg, 2000).

A case for teleost fish in the study of periconceptional stress

Hitherto, most experimental studies on periconception stress and its influences on long term health have been carried out in rodent models, but there is reason to suppose that the evolutionary mechanisms are evolutionarily conserved across the vertebrate subphylum and therefore that fish are also subject to such influences (Steenbergen, Richardson and Champagne, 2011). Previous work on tropical reef fish demonstrated that changes in temperature and CO_2 in the parental environment alters metabolic activity of the offspring, suggesting that fish can inherit environmental information via epigenetic mechanisms

(Donelson *et al.*, 2011; Miller *et al.*, 2012). These and similar experiments however concerned the effects of environmental manipulations maintained throughout the lifespan of the parents, and thus did not reveal whether transgenerational effects can be enacted by exposure during critical windows (e.g. spermatogenesis). Given the conservation of the vertebrate stress response, and parental effects of stress in the periconception period in rodents, it is reasonable to suppose that stress during similar critical windows is influential to the offspring of fish via similar mechanisms. Both guppies, *Poecilia reticulata* (Larsson *et al.*, 2002) and zebrafish, *Danio rerio* (Kamstra *et al.*, 2015; Schüttler *et al.*, 2017) have become models of reproductive and developmental toxicology, and are poised to act as models of developmental and transgenerational effects of stress. Both are small tropical or subtropical cyprinid fish which breed readily in the laboratory and have colonised wide geographic ranges in the wild. Zebrafish are native to eastern India (Spence *et al.*, 2007) while guppies to northern South America (Meyer and Lydeard, 1993).

Comparative reproductive biology of zebrafish and guppies in relation to their ecology

Although they are both small cyprinid fishes which occupy a range of habitats, zebrafish and guppies possess vastly different reproductive biologies. Both engage in courtship behaviour, but while zebrafish are egg-laying external fertilisers with relatively subtle sexual dimorphism, guppies on the other hand are viviparous (live-bearing) internal fertilisers with a striking degree of sexual dimorphism. Viviparity is thought to have evolved from the ancestral egg-laying strategy via progressive shortening of egg retention time (Blackburn, 1992). Internal fertilisation is a prerequisite for viviparity and confers an advantage to males in ensuring paternity and an advantage to females as it facilitates sperm storage, which can ensure a reliable supply of sperm when ova are ready to be fertilised. Perhaps the most obvious advantage of viviparity in relation to the ecology of guppies is that it negates the need to find suitable egg-laying sites, and thus is a considerable advantage in the often fast-flowing streams which guppies naturally inhabit (Meyer and Lydeard, 1993), where eggs that are laid may be susceptible to displacement by the current or exposure to predators. Furthermore, the lack of any need to find appropriate egg-laying substrate may have facilitated the colonisation of an extraordinary range of

habitat types (Thibault and Schultz, 1978). As a consequence of viviparity, fecundity is far lower than in egg-laying species, but females invest far more resources per offspring and produce larger offspring which are more competitive under resource-limiting conditions (Thibault and Schultz, 1978).

By contrast, the range of habitats occupied by zebrafish tend to comprise slow-moving or standing water bodies such as ponds and rice fields, and where they are found in streams these tend to have a low flow regime. Their habitats also tend to be characterised by an abundance of zooplankton and absence of large predatory fishes (Spence *et al.*, 2007). For these reasons, zebrafish habitats are well-suited to the ancestral strategy of egg-laying.

While guppies have gonosomal sex determination (i.e. sex chromosomes, Charlesworth 2018), sex determination in zebrafish has little known genetic basis and appears to have a strong environmental component, as in the laboratory sex ratio is highly influenced by food supply and growth rate (Spence *et al.*, 2007).

Guppies as models of gestational effects of stress

As a livebearing fish, the guppy presents an intriguing model for the effects gestational stress on offspring. Despite viviparity, guppies remain lecithotrophic, in that eggs contain all nutrition necessary for development, deposited into a yolk prior to fertilisation (Reznick, Callahan and Llauredo, 1996). Therefore, female guppies could be used to study possible epigenetic effects of gestational stress independent of nutritional influences. Guppies exhibit plasticity in resource allocation to their offspring under different food availability regimes, producing fewer but larger offspring if diet is restricted during egg provisioning (Reznick, Callahan and Llauredo, 1996). Although exposure to mild stress during the provisioning period has been found to induce behavioural alterations in the subsequent offspring (Eaton *et al.*, 2015), long-term effects of stress during the gestation period have not been studied experimentally. Exposure to endocrine-disrupting chemicals during gestation has been demonstrated to induce long-term effects on offspring phenotype in guppies (Kinnberg, Korsgaard and Bjerregaard, 2003; Shenoy, 2014), and so it is plausible that the physiological changes induced by gestational stress may induce similar long-term changes.

Zebrafish as models of transgenerational effects of stress

In contrast to guppies, zebrafish are egg-laying fish, the eggs of which are fertilised and develop as embryos externally. Because the embryo's chemical environment can be easily manipulated, they have found favour with developmental toxicologists, and also show promise as a model of foetal alcohol syndrome disorder (Baiamonte, Brennan and Vinson, 2015). They are ideal for studying the effects of pre-conception stress, as the maternal environment does not impose an influence during embryonic development. Developmental plasticity of the HPI axis has been previously demonstrated in response to embryonic (Ivy, Robertson and Bernier, 2017) and maternal environmental manipulations (Jeffrey and Gilmour, 2016a), and there is evidence for a maternal environmental component to the heritability of personality traits (Ariyomo, Carter and Watt, 2013).

As mentioned previously, oogenesis is continuous in adult teleosts in contrast to mammals, and thus they offer a system in which possible transgenerational influences resulting from disruption to early oogenesis may be studied independently of other gestational influences. However, despite external development, many other maternal factors could influence offspring phenotype. For instance, zebrafish embryos can be subject to the influence of maternal stress via deposition of cortisol into the oocytes, which has been demonstrated to exert measurable influences on offspring phenotypes, which may be mediated by epigenetic mechanisms in the early embryo (Faught, Best and Vijayan, 2016; Jeffrey and Gilmour, 2016a). Therefore, transgenerational effects of maternal stress do not necessarily require the stable transmission of epigenetic marks or noncoding RNA. By contrast, paternal effects of stress are more likely to be mediated by epigenetic factors carried in the sperm, and the externality of development, along with the short duration of spermatogenesis (only six days; Leal et al. 2009) makes the zebrafish a highly attractive system with which to study them.

So far, paternal effects mediated by miRNAs have been identified only in rodent models, with evidence of similar mechanisms existing in *Caenorhabditis elegans* (Grossniklaus *et al.*, 2013). Whether such mechanisms exist in distantly related vertebrates, such as fish, is not known, although non-genetic transgenerational phenomena associated with environmental stress have been observed in teleost fish (Miller *et al.*, 2012), and miRNAs are known to play an essential role in teleost spermatogenesis

(Babiak, 2014). Evidence that experimental manipulation of sperm competition and social status alter offspring life history and behavioural traits (Zajitschek *et al.*, 2014, 2017) suggest that environmental information can be transmitted in zebrafish sperm. However, paternal effects have not been investigated in the context of the stress response. If the mechanism of paternal environmental inheritance in teleost fish is similar to that which are beginning to be delineated in rodents, it would hint at the evolutionary significance of miRNA-mediated environmental inheritance (Grossniklaus *et al.*, 2013). Furthermore, zebrafish may be particularly sensitive to paternal effects given the previously-mentioned evidence that substantial paternal methylation can be inherited (Jiang *et al.*, 2013; Potok *et al.*, 2013). Although unusual compared to mammals, the inheritance of paternal methylation may be controlled by similar RNA-related mechanisms which seem to exist in mammals.

Conclusion and thesis aims

Psychological wellbeing and stress response physiology are intrinsically linked, and both epidemiology and experimental neuroscience have demonstrated that disrupted stress physiology underlies associations between psychological illness and stressful past experience. In a world subject to rapid technological and socioeconomic change, assessing the developmental and intergenerational influences of stress on long-term health may be more important than it ever was. While rodents have thus far been invaluable in efforts to characterise the influences of periconceptional stress and elucidate their underlying mechanisms, teleost fish are well-placed to complement or in some cases replace rodents in these efforts. However, the influences of periconception stress remain poorly characterised in teleost fish, and thus fundamental experimental work is necessary if their suitability as models of this paradigm are to be further evaluated. Such was the impetus for this thesis which has aimed to characterise the effects of different modes of parental stress during critical windows on the offspring of two model teleosts: zebrafish and guppies. In the case of guppies, the effects of stress during the gestation period were assessed with the hypothesis that gestation is a critical window during which embryonic offspring are susceptible to long-term effects of maternal stress. In zebrafish, the effects of paternal stress were investigated with the hypothesis that spermatogenesis is a critical window of susceptibility to intergenerational effects on offspring stress responses.

Chapter 2

Gestational alarm cue exposure stunts offspring growth in a live-bearing fish, *Poecilia reticulata*

Abstract

Exposure to elevated stress may have important consequences for the long-term fitness of animals, as well as for the fitness of their offspring via maternal effects. Guppies, for which predation represents an important environmental stressor, show a pronounced behavioural stress response following exposure to alarm cues extracted from the skin of conspecifics. We therefore used alarm substance as a model stressor to test the effects of daily stress during gestation on the growth, behaviour, and stress responses of subsequent offspring. Offspring from exposed mothers exhibited stunted growth in early postnatal development. Early growth rate was highly correlated with the mother's gestation length, which was shortened in response to alarm cue exposure. At maturity, offspring showed signs of weakened behavioural and endocrine stress response, but no significant alterations were detected and thus it could not be concluded that the effects of gestational stress persisted to adulthood. An additional experiment examined the influences of a different form of gestational stress, dietary restriction, which affected female reproduction differently (reduced brood size but did not affect gestation length) and did not significantly affect offspring growth, suggesting that the observed effects of alarm substance were not mediated by differences food consumption. Our findings suggest that shortening of the gestation period in stressful environments may have important downstream fitness consequences for the offspring of livebearing fish, corresponding with similar patterns observed in mammals, but that the effects of stress on reproduction and offspring may vary depending on the type and severity of the stressor.

Introduction

As aspects of the environment such as resources, competition, and predation are in constant flux, it is essential for organisms to respond plastically to transient environmental changes. Maternal effects present a set of mechanisms by which such environmental changes can also influence offspring, and can be either adaptive or maladaptive (Mateo, 2014). The guppy (*Poecilia reticulata*), a live-bearing poeciliid, is a popular model for the study of maternal effects particularly in the contexts of evolutionary biology (Reznick & Yang 1993) and environmental toxicology (Larsson *et al.*, 2002; Kinnberg, Korsgaard and Bjerregaard, 2003; Shenoy, 2014). Stress, defined as a condition of disrupted homeostasis that stimulates a stress response to enhance survival (Ord, Fazeli and Watt, 2017), can be an important source of maternal effects (Murray *et al.*, 2018). Stress responses in teleosts are characterised by increases in the glucocorticoid hormone, cortisol, which facilitates metabolic and behavioural changes necessary to restore homeostasis (Barton, 2006). While essential to respond to short-term changes, excessive activation of the stress response may lead to a maladaptive state (Prunet *et al.*, 2008). Examples of maternal effects has been demonstrated in the context of nutritional stress. For instance, in guppies, in which nutrition is provided to embryos via deposition in the yolk pre-fertilisation (Pires, Arendt and Reznick, 2010), mothers fed a restricted diet during yolk provisioning (prior to fertilisation) produce fewer, but larger offspring in the subsequent brood, reflective of plasticity in resource allocation to maximise offspring survival in resource-poor environments (Reznick and Yang, 1993; Reznick, Callahan and Llauredo, 1996). In a range of teleosts including guppies (Eaton *et al.*, 2015), zebrafish (Jeffrey and Gilmour, 2016b), and sticklebacks (Giesing *et al.*, 2011), exposure to other forms of stress such as social dominance or predation during egg provisioning has been associated with behavioural alterations in the offspring, which may be mediated by alteration of hormone content in the eggs which subsequently impact on development.

Predation threat can be considered an important form of stress for prey species (Clinchy, Sheriff and Zanette, 2013). Inevitably, small teleosts such as guppies exhibit well-characterised anti-predator behaviour, both individually and in groups (Kelley *et al.*, 2003). While guppies possess a highly developed visual system, they also rely on olfactory cues to detect and evade predation (Stephenson,

2016). Specifically, chondroitin fragments released from the skin upon damage are odorants which trigger innate anti-predator responses and comprise what is colloquially referred to as alarm substance (Mathuru *et al.*, 2012). A protocol for purification of alarm substance has been developed, and this purified form has been previously applied as a model stressor in experimental studies. Guppies and other small teleosts, such as the zebrafish, *Danio rerio*, typically respond to purified alarm substance by freezing (suppressing movement), remaining at low positions in the tank (Speedie and Gerlai, 2008; Egan *et al.*, 2009; Stephenson, 2016), and increasing their tendency to shoal (Nordell, 1998). Alarm substance also induces pronounced increases in cortisol in the zebrafish (Eachus *et al.*, 2017) and medaka (Mathuru, 2016) teleost models. Guppies reared in the presence of cues from a live predator have reduced cortisol levels (Fischer *et al.*, 2014), implying that long term exposure to increased predation threat elicits fundamental alterations to stress physiology.

Although Poeciliid fishes, to which guppies belong, are unique amongst teleost fish in that they are one of only 13 families to evolve vivparity (live-bearing) (Wourms, 1981), maternal effects of stress which originate from exposure during the gestation period have been less studied. However, predation stress has important life history consequences which may impact the fitness of the offspring via critical influences on gestation. For example, in response to repeated alarm substance exposure in mid-to-late gestation, female guppies shorten their gestation time (Evans, Gasparini and Pilastro, 2007), which likely represents an adaptive response to enhance maternal survival due to the detrimental effect of pregnancy on escape ability. Although maternal stress was not found to directly affect offspring performance traits, neonate swimming speed was negatively correlated with gestation length, suggesting maternal stress can influence the behaviour of young offspring, at least indirectly (Evans, Gasparini and Pilastro, 2007). Concordantly, in naturally high-predation environments, female guppies produce smaller, less well-developed offspring than their low-predation counterparts (Dial, Reznick and Brainerd, 2017).

There is also evidence that long term effects of gestational exposures on the offspring of live-bearing fish may arise directly from chemical changes that occur in-utero. For instance, gestational exposure to environmental endocrine disruptors induces morphological (Kinnberg, Korsgaard and Bjerregaard,

2003) and behavioural alterations (Shenoy, 2014) in adult guppy offspring, implying that exposure to altered physiological conditions (such as those induced by stress) in gestation affects long-term development. Despite guppies allocating all necessary resources to eggs pre-fertilisation, there is also evidence that offspring development is affected by maternal diet during the gestation period, as fecundity is influenced by resource availability post-egg provisioning (Reznick and Yang, 1993). While long-term effects of gestational nutrient stress have not been investigated, given that chemical changes in gestation can alter long-term development of guppies (Kinnberg, Korsgaard and Bjerregaard, 2003), it is possible that physiological changes associated with nutrient stress may induce alterations in a similar fashion.

Herein, we report the results of experiments investigating the influence of two forms of gestational stress (predation cues and dietary restriction) during gestation on guppy reproduction and offspring traits. While the effects of gestational predation stress have been investigated previously in young guppy offspring (Evans, Gasparini and Pilastro, 2007), the longer-term implications for offspring development and behaviour in adulthood have hitherto not. Here, we tested whether gestational stress had long-term developmental consequences for guppy offspring. In addition to assessing the effects of repeated alarm substance exposure on maternal behaviour and reproductive traits, we expanded on previous work (Evans, Gasparini and Pilastro, 2007) by comparing life history (growth), behaviour, and stress response phenotypes (behavioural and cortisol responses to alarm substance) of offspring derived from mothers exposed to alarm substance during gestation and non-exposed mothers. We predicted gestational predation stress would alter maternal reproductive traits as previously reported, and that changes in offspring phenotypes would reflect long-term consequences of gestational stress. By manipulating the diets of gestating mothers in a separate experiment, we were also able to evaluate whether different forms of stress affect reproduction and offspring life history in different ways, and also assess whether any effects of gestational stress by alarm exposure may have been mediated by differences in food consumption or demand (Clinchy, Sheriff and Zanette, 2013).

Materials and methods

Ethics statement

All animal work was carried out under a UK Home Office licence for the use of animals in scientific procedures.

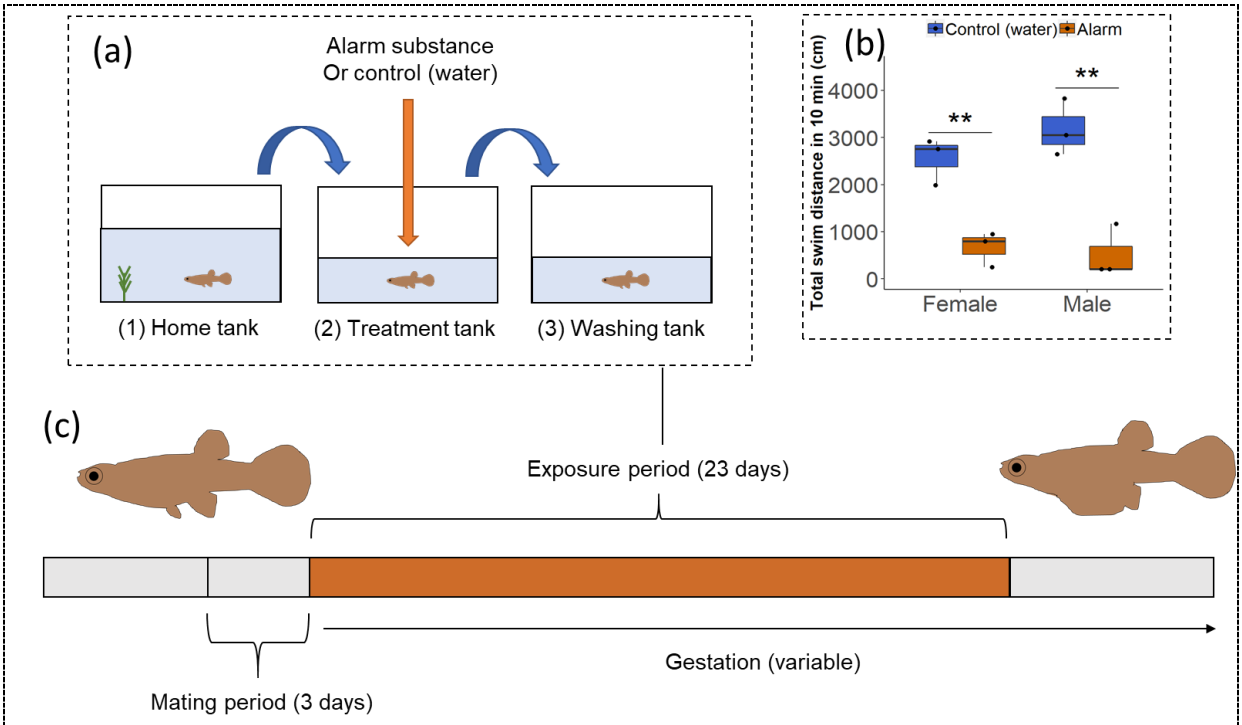

Figure 1. (a) Schematic of experimental treatment sequence. Individually-housed females were removed from their home tank daily and placed in a treatment tank to which alarm substance was added (or water as a control). Following 20 minutes of exposure, animals were transferred to a washing tank for 5 mins to remove traces of alarm substance before returning to their home tank. (b) The effect of alarm substance was validated by testing the effect of exposure on swim distance of individual males and females, in which it induced cessation of movement which we interpret as a behavioural stress response. (c) Timeline of experimental manipulation in relation to mating and gestation. After a three-day mating period to supply females with sperm, females were exposed to alarm substance (or control) daily as described in (a) for the first 23 days of the gestation period.

Animals and housing

We used wild-type Trinidadian guppies, descended from a population obtained from Leicester Botanical Gardens, United Kingdom, and reared in our laboratory for several generations. Fish were

housed in aquaria containing dechlorinated water at 27˚C, maintained under a 12:12h (light:dark) photoperiod, and fed ad libitum twice per day with brine shrimp (*Artemia salina*) naupilii or commercial flake food. A population of fry was reared, and males were removed as soon as a gonopodium became visible (~ 2 weeks of age), leaving females that had not been mated. Prior to commencing experiments, mature (> 4 months old) unmated female guppies (F0) were housed individually in 1L tanks (19 x 13 x 12 cm) for one month to allow for acclimation and to ascertain virginity. Each tank was supplied with an air stone and water was changed weekly. A plastic plant was provided for enrichment. Each female was weighed before the start of the experimental period. Wet weight was recorded by placing a beaker with 200 ml aquarium water on electronic scales and taring the scales before gently netting the fish into the beaker.

Mating

Prior to the commencement of experimental treatments, (or shortly after in the case of the diet experiment) virgin females were provided with a supply of sperm from mature male guppies taken from pre-selected stock (individuals of consistent size were selected). A different male was added to each tank on each of three consecutive days, in order to reduce any possible effect of sperm quality and increase the probability of mating. For the diet experiment, males were added approx. 2 hours after females were fed in the morning, and removed shortly before feeding the following morning, to prevent males from competing with females for food.

Alarm substance extraction and validation

Alarm substance was derived from mature female guppies based on established methods (Mathuru *et al.*, 2012; Schirmer, Jesuthasan and Mathuru, 2013). For every 3 mL of extract, five fish were euthanised, and 5-10 lacerations were made to the epidermis on both sides of each fish. All five fish were then placed in a single 50 mL tube with 3 mL water and gently shaken to release alarm substance. The water containing the extract was then removed, incubated at 95˚C for 16 hours, centrifuged, and the supernatant was extracted and filtered. Effectiveness of the extract to induce a measurable behavioural stress response was validated by measuring the locomotion of experimentally naïve adult

female and male guppies (total swim distance in 10 mins, see *Offspring behavioural stress response*) during exposure to 200 µL in 600 mL water. The prepared alarm substance was found to significantly impede swim distance in both male (two-sample T-test, $T^4 = 5.3$, $p < 0.01$) and female guppies ($T^4 = 5.6$, $p < 0.01$) (Fig. 1b).

Experimental manipulation: gestational predation stress (GPS) by exposure to alarm cue

A total of 12 females were selected for the experiment, out of which 6 were assigned randomly to the gestational predation stress treatment (GPS), and the other 6 as controls. After the three-day mating period, GPS females were exposed to alarm substance (200 µL in 600 mL) in separate exposure tanks for 20 minutes per day for 23 days (Fig. 1, a & c), such that both mothers and developing progeny were exposed. Control females were placed in separate tanks and exposed to aquarium water only for 20 mins per day for 23 days, simultaneously to alarm-treated females. Individuals were not visible to each other during treatment. One control female died during the experiment, and another control failed to produce a brood after 60 days, so that in total, six GPS and four control broods were derived. Brood sizes ranged from 4 to 30 fry (Table 1). Behavioural tests were carried out seven days following the final alarm substance exposure (see *Behavioural tests*).

Experimental manipulation: gestational dietary restriction

To obtain measured quantities of food, newly hatched brine shrimp nauplii were poured through a fine sieve to remove excess water until they formed a thick paste. This paste was then loaded into a 250 ul syringe, and measured quantities (see below) were then either administered directly to the water or deposited into 1.5ml Eppendorf tubes with 0.25 ml water and frozen until needed. Females were assigned randomly to one of two diet regimes and fed with quantified amounts of brine shrimp paste (50 ul for high food diet and 10 ul for low food diet, respectively) once per day for a total of 31 days. Food quantities were derived from Reznick *et al* (1996), who reported that the high food level was close to *ad libitum*, while the low food level was sufficient to sustain reproduction in mature female guppies. These diets were maintained from two days before mating (see *Mating*), until 26 days after mating to

cover the average gestation length (Reznick *et al*, 1996). After this period, the diet of all females was restored to *ad libitum* brine shrimp. Eight females comprised each of the high and low diet groups. One high diet female and 3 low diet females failed to produce litters after 65 days, such that broods were derived from 7 high diet and 5 low diet females. Females from the high and low diet groups which produced broods did not differ in wet weight at the start of the experiment (two-sample T-test, $T^{8.7} = 0.17$, $p = 0.86$).

Offspring rearing and growth monitoring

For the GPS experiment, on the day of parturition up to 12 offspring were taken randomly from each brood for further rearing. To control for population density effects, each fry was housed in an individual compartment of a breeding trap (20 x 9 x 10 cm) divided into three equal-sized compartments. Four such divided breeding traps were kept in trays (45 x 29 x 13 cm) containing approximately 5 L water that could flow freely into each compartment through small holes in the sides of each breeding trap (Fig. 2). Each tray was supplied with a sponge filter, and water was changed weekly. Fry were fed *ad libitum* as previously described. After 21 days, partitions were removed so that juveniles from the same brood could interact in the breeding traps in groups of 2-3 to provide opportunity for social interaction and greater space for movement. Juveniles were checked regularly for the appearance of sexual characteristics (e.g. presence of gonopodium in males), and fish of different sexes were placed in separate compartments with their same-sex siblings; males were housed mostly in groups of 2-3 and females in groups of 2-4. No broods contained fewer than two males, although one male was housed singly because its' male sibling had initially been misidentified as a female. Offspring mortality during rearing was negligible.

For the diet manipulation experiment, offspring were not housed individually as described, but if necessary, were separated into groups of no more than eight per 1L tank (19 x 13 x 12 cm).

Offspring size measurements

On the day of parturition, a litter was gently netted into a petri dish containing a shallow film of water (approx. 15 ml). The petri dish was placed on a sheet of graph paper resting on a sheet of opaque glass,

suspended over a light to ensure adequate contrast between fish and the graph paper. The petri dish was then photographed. This was repeated for three randomly-selected offspring from each brood every three days for the first 15 days post-parturition. Photos were imported into ImageJ to facilitate accurate size measurements, using the graph paper for calibration. Body area (excluding tail) was measured from photographs, and the mean body area was taken for each brood for each measurement day.

At approximately 60-70 days post parturition, offspring were weighed individually in a plastic beaker containing 20 mL water, using a digital balance.

Behavioural tests (GPS experiment only)

Seven days after the final exposure to alarm substance, adult females were tested for movement activity and anxiety-like behaviour using the open field test (Rehnberg, Smith and Sloley, 1987; Ariyomo, Carter and Watt, 2013). For each trial, an individual fish was gently netted into a rectangular tank (40 x 25 x 25 cm) containing 3 L of water. The test tank was covered on all sides with paper to prevent external visual stimuli. Each fish was video-recorded from above for 5 mins using a Panasonic HC-X920 camcorder. Water was replaced between each trial. The same testing procedure was performed on the offspring in a smaller tank (25 x 25 x 25 cm) at 50-60 days post-parturition, and up to three males and three females from each parent were used. Videos were scored manually for swimming activity (number of line crossings on an 8 x 8 grid covering the entire tank area) and thigmotaxis (% time spent in the outer grid squares of the tank), a common measure of anxiety-like behaviour.

Offspring behavioural stress response (GPS experiment only)

To examine the effect of gestational stress on the behavioural response of F1 adult offspring, we measured the effect of alarm substance exposure on swim distance. From each brood, up to two male and two female (three in one case) offspring were exposed to alarm substance (200 µL in 600 mL), and another up to two of each sex were exposed to aquarium water only (control) in tanks (19 x 13 x 12 cm). See Table 2 for exact numbers exposed from each brood. For broods with more than two offspring of a given sex, animals were selected randomly irrespective of whether they had been previously tested in the open field test. Alarm and control exposures were conducted simultaneously for 10 minutes, after

which fish were immediately euthanised in 3g/L MS-222, snap-frozen in liquid nitrogen and stored at -80˚C for subsequent cortisol extraction. Behaviour was recorded during exposures from above using a using a Panasonic HC-X920 camcorder, and total swim distance was measured from videos using ZebraLab automated observation software (ViewPoint Life Sciences, Inc.).

Offspring whole-body cortisol (GPS experiment only)

Cortisol levels were measured from female and male offspring following treatment with alarm substance or aquarium water. Whole fish were weighed and homogenised using a pellet mixer in 2 mL Eppendorf tubes with 1 mL of water. Homogenate was mixed with 1 mL diethyl ether (Sigma), centrifuged, and the ether layer, containing cortisol, was eluted. Ether was then evaporated, cortisol was reconstituted in 1 mL phosphate-buffered saline and stored at -20˚C. Enzyme-linked immunosorbent assay (ELISA) was used to quantify the cortisol concentration of samples, according to a previously published protocol (Yeh, Glöck and Ryu, 2013), with separate assays run for female and male samples. Samples were run in duplicate and cortisol standards in triplicate. The mean intra-assay coefficient of variation was 7.2%. Duplicate measurements were averaged for each sample and whole-body cortisol was estimated according to the weight of the fish.

Statistical analyses

All statistical tests were conducted using 'R' version 3.4.0 (R Core Team, 2017). T-tests were used to assess statistical significance of differences in reproductive traits (gestation length, fecundity). For analyses of offspring parameters, we performed linear mixed effects models fit by residual maximum likelihood (REML), using the 'lme4' package (Bates et al 2014), with nested random effect terms, where applicable. Significance of mixed model terms was evaluated using the 'lmerTest' (Kuznetsova et al 2017) and 'pbkrtest' packages (Halekoh et al 2014) using T-tests and F-tests with Kenward-Roger approximation of degrees of freedom. Response variables were log-transformed in the case of non-normal distributions.

Models with second-order polynomial regression were used to evaluate the effect of maternal treatment on offspring growth, with 'offspring age', 'treatment', and 'age x treatment' as fixed effects terms, and

‘mother ID’ as a random effect term. Inclusion of the second order polynomial was found to improve the model fit as indicated by lower AIC scores for both GPS and diet experiment models.

For analyses of adult offspring wet weight at maturity and manual behavioural parameters, offspring sex was included as a fixed term in the models, with mother ID always included as a random effect term. For offspring wet weight in response to maternal diet, sex the interaction term ‘maternal x sex’ was included as it resulted in a lower AIC score compared to when sex was included only as an additive effect. For behavioural stress response (total swim distance following alarm or control exposure), sex, offspring treatment (alarm or control), maternal treatment (control or GPS), and the interaction between the latter two (treatment x maternal) were fixed effect terms, while mother ID was again a random term. Inclusion of sex as an interaction term did not improve model fit as judged by AIC score for any of the aforementioned models. For analysis of offspring cortisol levels following alarm or control treatment, the data from each sex were analysed with separate models due to male and female samples being measured in separate ELISAs. Post-hoc T-tests using the ‘emmeans’ package were used to test the effect of alarm substance on control and GPS offspring separately.

Table 1. Weight, gestation length, and brood sizes of females which produced broods in two experiments.

Exp.	Female	Weight (g)	Treatment	Gest. length (days)	Brood size
GPS	1	0.51	Control	56	4
	2	0.41	Control	64	22
	3	0.34	Control	51	15
	4	0.56	Control	49	20
	5	0.53	GPS	53	25
	6	0.59	GPS	49	23
	7	0.52	GPS	40	30
	8	0.46	GPS	25	18
	9	0.55	GPS	49	22
	10	0.49	GPS	29	6
Diet	1	0.54	High Diet	50	17
	2	0.59	High Diet	31	16
	3	0.53	High Diet	45	14
	4	0.54	High Diet	53	2
	5	0.53	High Diet	37	13
	6	0.73	High Diet	54	18
	7	0.73	High Diet	44	18
	8	0.71	Low Diet	64	3
	9	0.46	Low Diet	51	10
	10	0.58	Low Diet	44	5
	11	0.55	Low Diet	53	2
	12	0.64	Low Diet	31	8

Table 2. Numbers of offspring derived from each mother in the GPS experiment, the numbers reared, and the numbers of females (F) and males (M) used for each type of measurement. SR refers to stress response test, where the two numbers within the brackets denote the number used as controls and the number exposed to alarm substance, respectively.

Mother	Treatment	Brood size	No. reared	No. weighed		No. tested (OF)		No. tested (SR)	
				F	M	F	M	F	M
1	Control	4	4	2	2	3	1	2(1/1)	2(1/1)
2	Control	22	12	3	3	3	3	4(2/2)	3(2/1)
3	Control	15	12	6	2	3	2	4(2/2)	2(1/1)
4	Control	20	12	6	4	3	4	4(2/2)	4(2/2)
5	GPS	25	12	9	2	3	2	4(2/2)	4(2/2)
6	GPS	23	12	7	2	3	2	4(2/2)	2(1/1)
7	GPS	30	12	8	4	3	3	6(3/3)	4(2/2)
8	GPS	18	12	10	2	3	2	4(2/2)	2(1/1)
9	GPS	22	12	3	2	3	2	2(1/1)	2(1/1)
10	GPS	6	6	3	2	3	1	4(2/2)	1(0/1)

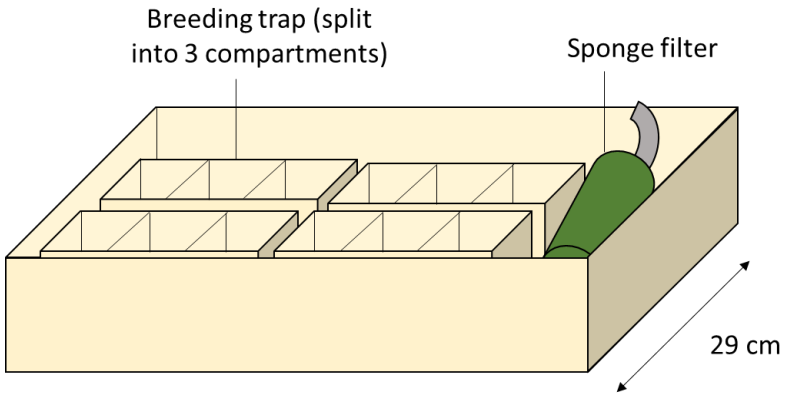

Figure 2. Representative schematic of housing tray in which guppy offspring were reared. Individuals were housed in separate compartments of breeding traps divided using plastic inserts.

Table 3. Sample sizes, response variables, fixed and random effect terms for each mixed effects model. Interactions are denoted by 'x'. For analyses, in which multiple individual offspring per parent were tested, the number outside the brackets is the number of broods and the number inside the brackets the number of offspring.

Model / fixed effect terms	Random	Group	N(n)	Response variable
1 & 2) Maternal x Age^2	Mother ID	Control	4	Brood mean body area (log scale)
		GPS	6	
		High diet	7	
		Low diet	5	
2) Maternal + sex	Mother ID	Control	4(28)	F1 wet weight (mg)
		GPS	6(54)	
3) Maternal x sex	Mother ID	High diet	7(30)	F1 wet weight (mg)
		Low diet	5(19)	
4 & 5) Maternal + sex	Mother ID	Control	4(21)	F1 thigmotaxis (%)
		GPS	6(30)	F1 line crossings
6) Maternal x Treatment + sex	Mother ID	Control-control (water)	4(13)	F1 swim distance (cm)
		Control-alarm	4(12)	
		GPS-control (water)	6(17)	
		GPS-alarm	6(18)	
7) Maternal x Treatment	Mother ID	Control-control (water)	4(7)	F1 cortisol (ng/g body weight) (females)
		Control-alarm	4(7)	
		GPS-control (water)	6(9)	
		GPS-alarm	6(9)	
8) Maternal x Treatment	Mother ID	Control-control (water)	4(9)	F1 cortisol (ng/g body weight) (males)
		Control-alarm	4(6)	
		GPS-control (water)	5(7)	
		GPS-alarm	6(8)	

Results

Reproductive and life history parameters

GPS was associated with shortened gestation length (two-sample T-test, $T^{7.9} = 2.4$, $p = 0.041$) (Fig. 3b), but there was no effect on fecundity as measured by number of offspring per unit body weight ($T^{7.2} = 1.48$, $p = 0.18$) (Fig. 3c). While there was no effect of gestational diet on gestation length (Fig. 3f), females fed the restricted diet produced significantly fewer fry per unit body weight ($T^{9.7} = 2.8$, $p = 0.02$) (Fig. 3g).

GPS offspring were on average 0.97 ± 0.27 mm^2 smaller than control offspring during the first two weeks post-parturition, and while control offspring exhibited essentially linear growth, the trajectory of GPS

offspring growth was noticeably more curve-shaped (Fig. 3a). A linear mixed model with maternal treatment and time (quadratic term) as fixed effects and mother as a random effect to account for repeated measurements revealed a significant effect of treatment ($F^{1,7.8}$ = 12.75, p = 0.007), and a significant age x treatment interaction ($F^{2,36}$ = 4.27, p = 0.02). The difference in growth trajectory appeared to be driven by stunted growth in the first few days post-parturition. Offspring size throughout early development was not affected by gestational dietary restriction (Fig. 3e), however there was a marginally non-significant interaction between maternal diet and the first order polynomial of age ($T^{45.1}$ = 1.84, p = 0.07). Although there remained a trend towards smaller offspring in GPS broods, offspring weight at maturity was unaffected by maternal GPS ($F^{1,8.3}$ = 2.05, p = 0.19) (Fig. 3d). Offspring weight at maturity was similarly unaffected by dietary restriction ($F^{1,6.9}$ = 0.05, p = 0.83), however there was a noticeable trend towards heavier males in low diet broods (Fig. 3h) which was reflected by marginally non-significant interaction between maternal diet and sex ($F^{1,34.2}$ = 3.1, p = 0.09).

To assess the extent to which the difference in early growth associated with GPS was driven by gestation length, we assessed the relationship between gestation length and brood mean growth rate for the period between day 0 and either day 3 or 6 post-parturition (as neither day 3 nor 6 featured available data for all broods) across both alarm cue and diet experiments. There was found to be a positive linear relationship between growth rate and gestation length (Pearson's r = 0.61, p = 0.002) (Fig. 4).

Table 4. Results of two linear mixed effects models describing the effect of 1) maternal gestational predation stress (GPS) and 2) gestational low diet on offspring size measured over the first two weeks post-parturition. Models were fitted to log-transformed data. Both F-test and T-test results of fixed effect terms are shown, with F values derived from the effect of adding a term last to the model, while T values derive from comparing the parameter estimate to the intercept in the absence of other factors. Variance and standard deviations of random effect terms are shown.

GPS experiment (N = 10 broods)								
Fixed effects	ndf	ddf	F	p	Estimate	SE	T	p
Intercept		7.76			1.86	0.03	57.23	< 0.001
Treatment (GPS)	1	7.84	12.75	0.01	-0.15	0.04	3.57	0.01
Age^2	2	35.83	178.49	< 0.001				
Age^1		35.30			1.91	0.15	13.14	< 0.001
Age^2		36.24			-0.07	0.14	0.49	0.63
Treatment x Age^2	2	35.83	4.27	0.02				
Treatment x Age^1		35.54			-0.30	0.19	1.62	0.12
Treatment x Age^2		36.13			0.45	0.19	2.40	0.02
Random effects	Variance	SD						
Mother	0.003	0.051						
Residual	0.008	0.090						
Diet experiment (N = 12 broods)								
Fixed effects	ndf	ddf	F	p	Estimate	SE	T	p
Intercept		10.02			1.79	0.04	40.64	< 0.001
Diet (low diet)	1	9.96	1.29	0.28	0.08	0.07	1.14	0.28
Age^2	2	45.18	213.53	< 0.001				
Age^1		45.29			1.67	0.12	14.28	< 0.001
Age^2		45.38			-0.05	0.12	0.47	0.64
Diet x Age^2	2	45.18	2.03	0.14				
Diet x Age^1		45.14			0.33	0.18	1.84	0.07
Diet x Age^2		45.21			0.15	0.18	0.85	0.40
Random effects	Variance	SD						
Mother	0.012	0.109						
Residual	0.008	0.087						

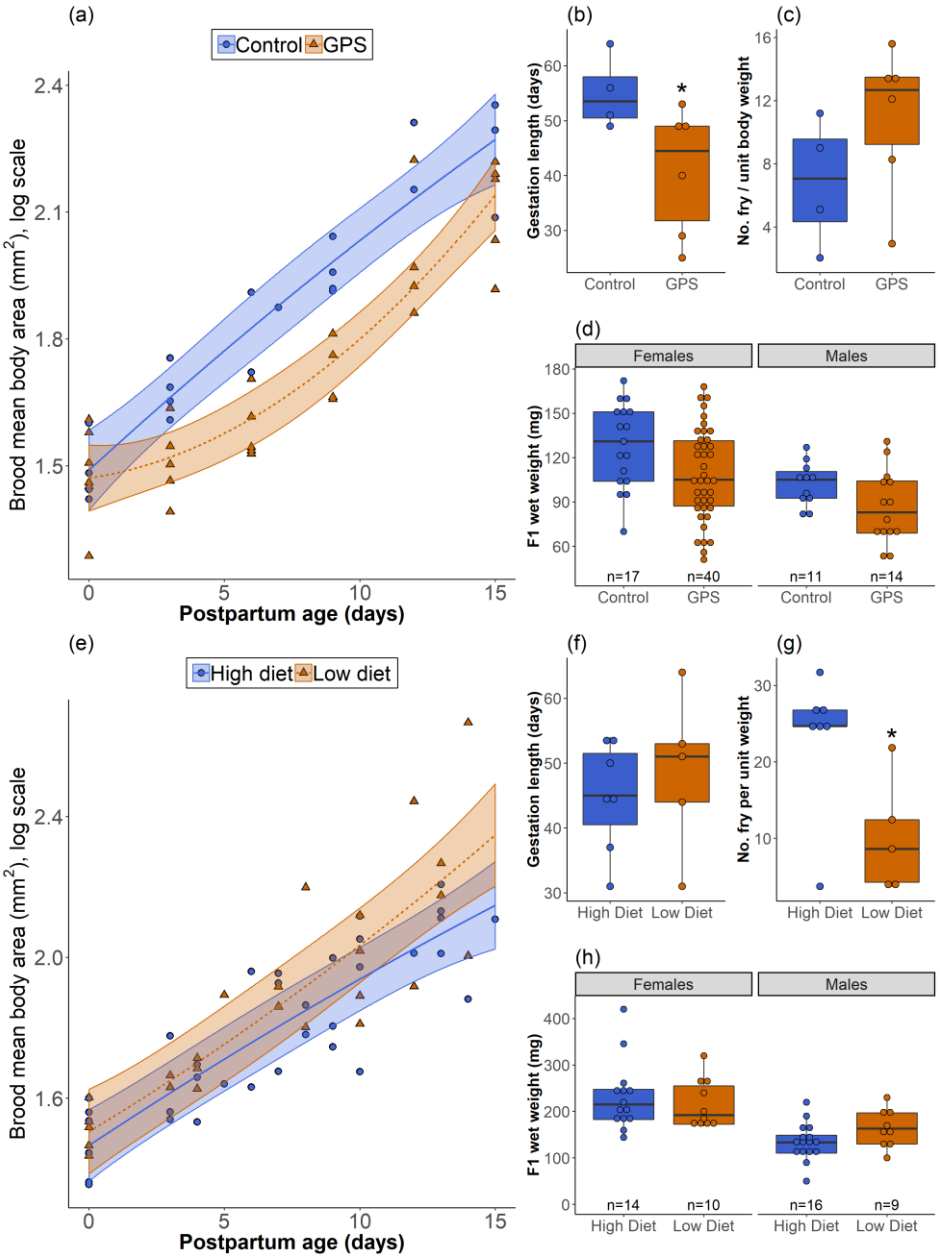

Figure 3. Maternal reproductive traits and offspring growth in response to gestational predation stress (GPS) and gestational diet manipulation. (a) Body size (brood mean body area, excluding tail region, log-transformed) measured from days 0-15 postpartum for control (blue circles) and GPS broods (orange triangles). Curve fits with 95% CIs were derived from linear mixed effects models with second-order polynomial regression. (b) and (c) gestation length in days, and fecundity (no. offspring per gram body weight), respectively for control (blue) and GPS (orange) mothers. (d) Wet weight (mg) of adult female (left) and male offspring (right) from control (blue) and GPS (orange) offspring, measured between 50-70 days postpartum. (e-h) identical paramaters as shown in (a-e) but comparing high diet (blue) with low diet (orange) treatments. * $p < 0.05$, two-sample T-test.

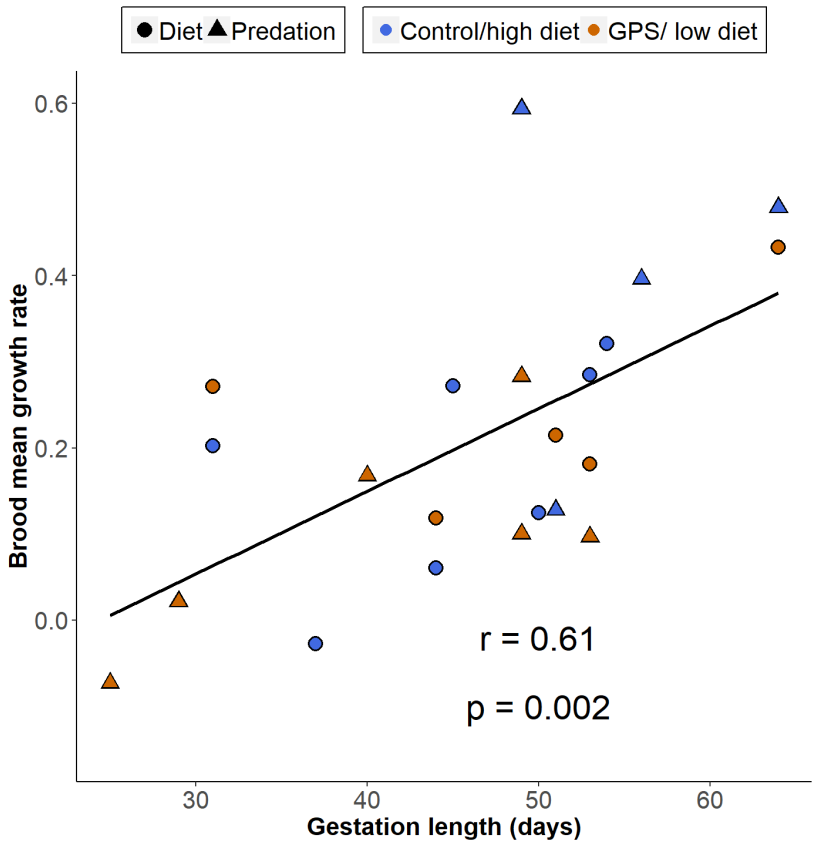

Figure 4. Brood mean growth rates (proportional increase in body area) between days 0-6 postparturition plotted against maternal gestation length (days) for 22 broods from four treatment groups across two experiments, with the colour and shape of points corresponding with the treatment group and experiment, respectively. Regression line, Pearson's correlation coefficient and corresponding p value are shown.

Behavioural tests

One week following the final alarm exposure, thigmotaxis was significantly reduced in F0 females from 71.37% to 40.94% (two-sample T-test, $T^{7.6} = 4.9$, $p < 0.01$), but there was no effect on swimming activity as measured by number of line crossings ($T^{6.9} = 0.14$, $p = 0.891$). There did not appear to be any marked differences in these parameters in the mature offspring, however (Fig. 5). We analysed offspring behavioural responses using linear mixed models with maternal GPS and offspring sex as fixed effect terms (non-interacting) and mother as a random term. We subsequently confirmed no significant effect of maternal GPS on thigmotaxis (T-test with Kenward-Roger approximation of df, $T^{7.36} = 1.1$, $p = 0.306$) or line crossings ($T^{8.34} = 0.37$, $p = 0.719$).

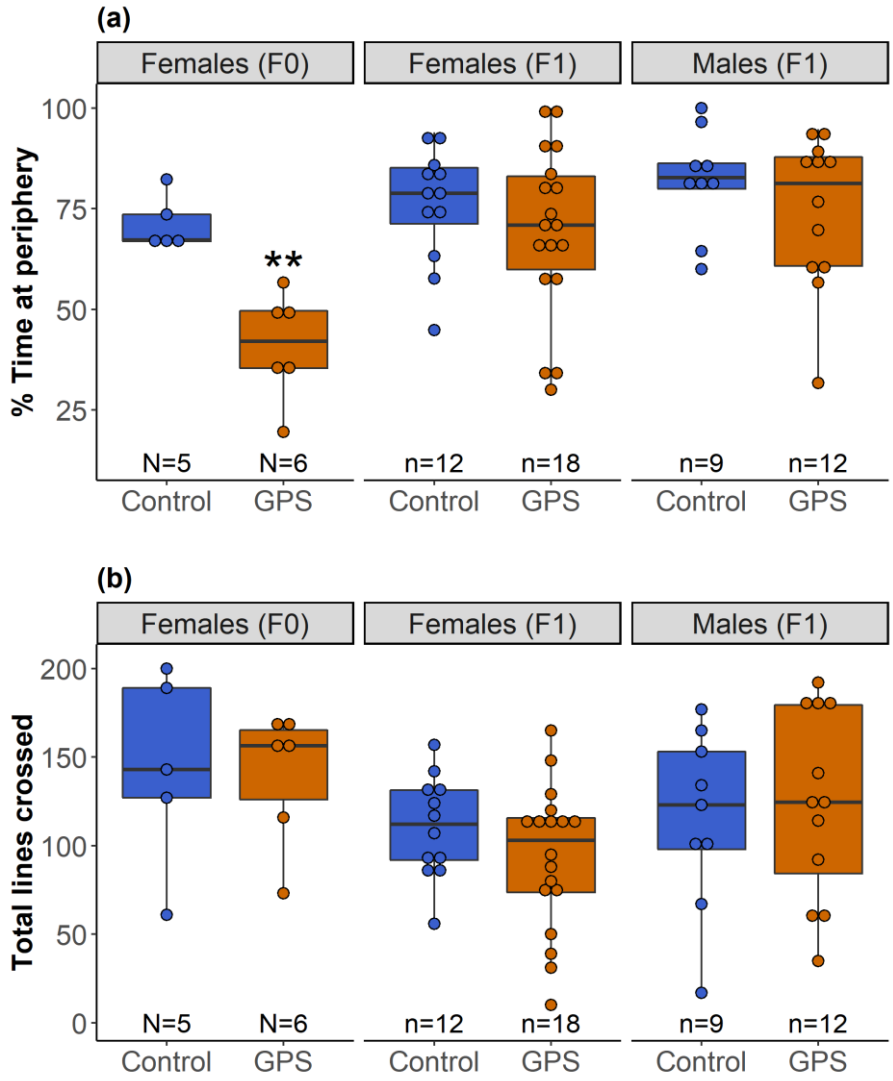

Figure 5. Open field test performance measurements in F0 females following 23 days of exposure to gestational predation stress (orange), or control (blue), and their subsequent female and male mature offspring. (a) Thigmotaxis, measured as the % time spent in the periphery of the test tank within the 5-minute observation period. (b) Movement activity measured as the total number of lines crossed on a grid during the 5-minute observation period. For F0, 'N' represents the number of biological replicates (mothers), while for F1 'n' represents the number of pseudoreplicates (offspring) in each group, derived from 4 control and 6 GPS mothers. * $p < 0.05$, two-sample T-test. Data were collected by Kelle Holmes.

Offspring behavioural stress response

Swim distance was generally lower in offspring exposed to alarm substance (Fig. 6), with control offspring showing a reduction of 461(±237) cm and GPS showing a smaller reduction of 342(±201) cm. A linear mixed effects model revealed an overall significant effect of offspring alarm treatment ($F^{1,48.2} = 6.68$, $p = 0.013$), but no effect of maternal treatment ($F^{1,6.89} = 0.009$, $p = 0.928$) or interaction between the two ($F^{1,48.2} = 0.146$, $p = 0.704$). Post-hoc T-tests were used to assess the effect of alarm substance on control and GPS offspring separately. In control offspring, the reduction in swim distance in response to alarm substance was marginally non-significant, ($T^{48.1} = 1.94$, $p = 0.058$) while in GPS offspring it was not significant ($T^{48.4} = 1.71$, $p = 0.095$). Sex had a significant additive effect, with males travelling on average 441.8(±155.6) cm more than females in the absence of alarm treatment.

Table 5. Results of a linear mixed effects model describing the effect of gestational predation stress (GPS) and offspring alarm treatment on swim distance in 10 mins. Both F-test and T-test results of fixed effect terms are shown, with F values derived from the effect of adding a term last to the model, while T values derive from comparing the parameter estimate to the intercept in the absence of other factors. Variance and standard deviations of random effect terms are shown. n = 60 offspring from 10 parents (4 control and 6 GPS).

Fixed effects	ndf	ddf	F	p	Est	SE	T	p
Intercept		22.55			1161.4	196.34	5.92	< 0.001
Treatment (Alarm)	1	48.22	6.70	0.01				
Treatment (Alarm)		48.09			-461.03	237.49	1.94	0.06
Maternal (GPS)	1	6.89	0.01	0.93				
Maternal (GPS)		17.74			-77.26	244.39	0.32	0.76
Sex (Male)	1	49.49	0.15	0.01	441.80	155.62	2.84	0.01
Treatment x Maternal	1	48.20	0.15	0.70	118.95	310.95	0.38	0.70
Random effects	Variance		SD					
Mother	23177		152.2					
Residual	350462		592					

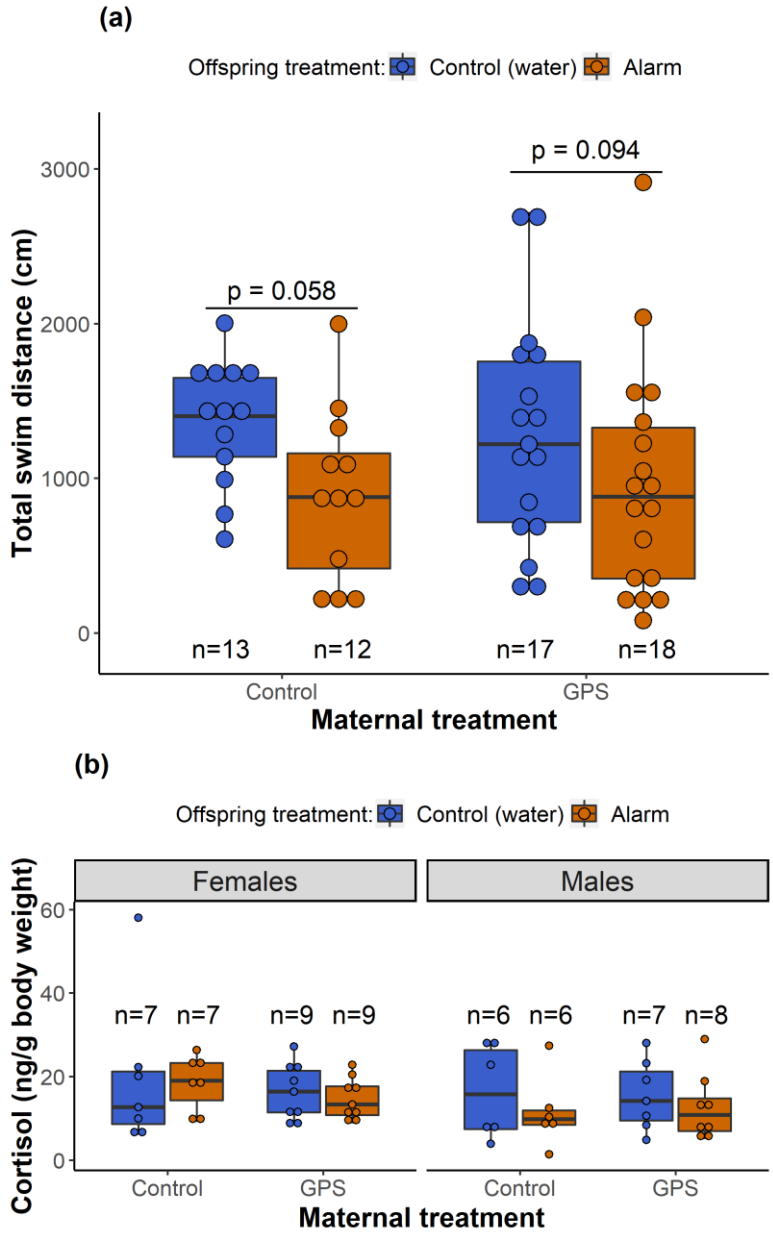

Figure 6. Stress response metrics of mature offspring derived from control and gestational predation stress (GPS) broods. (a) Total distance travelled over 10 minutes of exposure to either alarm substance (orange) or water as a control (blue) in control and GPS offspring. (b) Whole body cortisol levels (ng/mg body weight) following exposure to alarm substance (orange) or water as a control (blue) for control and GPS offspring. The cortisol plot shows an outlier in the female control – control (water) group which was removed prior to statistical analysis. 'n' denotes the total number of pseudoreplicates (offspring) per group, which derived from 4 control and 4 GPS mothers. P values derive from post-hoc T-tests of a linear mixed effects model with Kenward-Roger approximation of degrees of freedom.

Offspring whole-body cortisol

One prominent outlier sample in the female control – control (water) category which sowed abnormally high cortisol was removed prior to statistical analyses. After outlier removal, alarm treatment increased cortisol in control female offspring by 5.34 ± 2.74 ng/g, but there were no noticeable increases in the other groups. A mixed effects model fitted for female offspring revealed no overall significant effect of alarm treatment on the offspring ($F^{1,19.2} = 1.08$, $p = 0.311$), no effect of maternal GPS ($F^{1,7.7} = 0.03$, $p = 0.878$), but a marginally non-significant interaction between offspring alarm treatment and maternal GPS ($F^{1,19.2} = 3.78$, $p = 0.067$). Post-hoc T-tests revealed the increase in cortisol in control females exposed to alarm substance to be marginally non-significant ($T^{19.3} = 1.95$, $p = 0.066$), while there was no effect of alarm substance on GPS females ($T^{19.1} = 0.7$, $p = 0.491$). For males there was no effect of alarm treatment ($F^{1,16.2} = 1.25$, $p = 0.281$), maternal GPS ($F^{1,6.2} = 0.001$, $p = 0.973$), or treatment x maternal interaction ($F^{1,16.2} = 0.098$, $p = 0.758$).

Table 6. Results of a linear mixed effects model describing the effect of gestational predation stress (GPS) and offspring alarm treatment on whole body cortisol levels. Both F-test and T-test results of fixed effect terms are shown, with F values derived from the effect of adding a term last to the model, while T values derive from comparing the parameter estimate to the intercept in the absence of other factors. Variance and standard deviations of random effect terms are shown. n = 31 female offspring from 10 mothers (4 control and 6 GPS).

Fixed effects	ndf	ddf	F	p	Est	SE	T	p
Intercept		13.55			13.24	2.83	4.67	< 0.001
Treatment (Alarm)	1	19.23	1.08	0.31				
Treatment (Alarm)		19.34			5.34	2.74	1.95	0.07
Maternal (GPS)	1	7.73	0.03	0.88				
Maternal (GPS)		13.54			2.98	3.66	0.81	0.43
Treatment x Maternal	1	19.23	3.78	0.07	-6.95	3.58	1.94	0.07

Random effects	Variance	SD
Mother	15.42	3.93
Residual	23.81	4.88

Discussion

Exposure to elevated stress, particularly in the context of predation, is increasingly recognised to have important implications for reproduction and offspring health in a range of species (Clinchy, Sheriff and Zanette, 2013). Using a purified form of conspecific-derived alarm cue to simulate elevated predation in the environment of gestating female guppies, our findings are consistent with this overall paradigm, and furthermore confirm a previously-reported finding that guppies accelerate parturition in response to perceived increase in predation (Evans, Gasparini and Pilastro, 2007). Although we did not measure cortisol levels in gestating females exposed to alarm substance and thus could not verify that they were subject to physiological stress, it was evident from (1) the dramatic behavioural responses in naïve animals, (2) the shortening of gestation time in females, and (3) the alteration in thigmotactic behaviour in females after daily exposure, that physiological and neurological changes took place in the animals. The third observation, in particular suggests that daily exposure to alarm substance induced a sustained alteration to neurocircuitry, given that the observation was made seven days after the final exposure to the stimulus.

GPS offspring were smaller throughout early postnatal growth, and the significant treatment x age interaction indicates that GPS offspring grew slower during this early period. As GPS mothers had shorter gestation times and maternal gestation length positively correlated with early growth rate, it can be inferred that while early growth retardation was a consequence of gestational stress, this was driven by accelerated parturition. A plausible explanation for this is that due to earlier release into open water and subsequent resource expenditure on swimming, GPS offspring incurred a greater metabolic deficit at an earlier developmental stage, when yolk resources would otherwise be devoted to growth and other aspects of development. Females can enhance offspring swimming performance by delaying parturition to allow further intrauterine development (Shine and Olsson, 2003; Evans, Gasparini and Pilastro, 2007), and it could also be inferred that reduced growth rate occurs because offspring with restricted intrauterine development time are less effective foragers due to having less well-developed sensory systems at birth. However, that there was no difference in body size in adult offspring implies that some degree of compensatory growth occurred in GPS offspring (Auer, 2010).

Interestingly, we found that a different form of stress – dietary restriction – affected female reproduction differently to GPS, in that gestation length was unaffected but brood size was reduced, confirming an observation by Reznick & Yang (1993), thus suggesting that the dietary manipulation was sufficient to induce physiological changes. Apart from a slight upward curvature which appeared to be largely driven by a single brood, there was no discernible effect of dietary restriction on offspring growth, suggesting that the observed effects of GPS were not driven by behaviourally-mediated differences in food consumption or increased physiological demand for food (Clinchy, Sheriff and Zanette, 2013). Although the lack of effect of dietary restriction is not surprising given that all the nutrition necessary for embryonic development is supplied to guppy eggs prior to fertilisation, it is nonetheless interesting that offspring escaped deleterious effects given the state of physiological stress likely induced by dietary restriction (e.g. as observed in mammals, Seckl 2004). We concede however that, other than the decreased brood size, there was no evidence that females in the low diet condition experienced elevated stress. It is possible that abortion acts as a means by which mothers can reclaim nutrients, protecting themselves from nutritional stress and protecting their remaining offspring from physiological influences that may otherwise arise from it. However, it is considered unlikely that resorption of embryos bestows a net nutrient benefit to females, given that the ovarian follicle is not likely to act as an efficient gut and thus energy gained from resorption is unlikely to offset energy spent in the process (Meffe and Vrijenhoek, 1981). It is more likely, therefore, that aborted embryos are ejected and possibly consumed by females afterwards. Therefore, abortion of offspring could be considered a manifestation of adaptive plasticity in reproductive output which enables female guppies to maintain the quality of surviving offspring in the face of resource deprivation.

Mothers exposed to alarm substance for 23 days showed significantly reduced thigmotaxis, which would suggest lowered anxiety-like behaviour. This is counter-intuitive, as it would be expected that any adaptive responses to simulated predation would be in the direction of increased anxiety or reduced boldness. The reduction in thigmotaxis may reflect habituation of the stress response apparatus following repeated exposure to a stressor – a phenomenon which arises due to plasticity of the hippocampus (McEwen, 2006; Grissom and Bhatnagar, 2009). Although maternal behaviour was

affected, no such changes in baseline anxiety-like behaviour were found in the offspring, suggesting that gestational chronic stress does not have a major impact on long-term neurodevelopment of the offspring which affected their behaviour under standard test conditions.

We tested offspring from control and GPS mothers for their behavioural and cortisol responses to alarm substance to assess whether gestational stress influenced the stress response capacities of the offspring. Marked suppression of swim distance following alarm substance exposure was observed in both experimentally naïve animals and in control offspring, consistent with previously published observations (Stephenson, 2016). Interestingly, this response was noticeably weaker in GPS offspring despite a larger sample size than control offspring, implying a lack of sensitivity to the stressor. Subsequently, it could be inferred that maternal stress during gestation impaired stress responses in GPS offspring, possibly reflective of developmental trade-offs associated with compensatory growth (e.g. Arendt, 2003; Arendt et al., 2001; Auer et al., 2010) or long-term consequences of differential endocrine environment experienced in-utero (Kinnberg, Korsgaard and Bjerregaard, 2003; Shenoy, 2014). We detected a marginally non-significant interaction between offspring alarm treatment and maternal GPS on female offspring cortisol, reflecting the slight increase in cortisol in control females following alarm treatment while GPS females showed no discernible response, concordant with the noticeably weaker behavioural response. However, as there were no significant interactions between maternal and offspring treatments for either swim distance or cortisol, it cannot be concluded that alarm treatment induced changes in the offspring stress responses. We did not detect an effect of alarm substance on male cortisol levels, which is perplexing. However, due to the higher levels of movement in non-exposed animals and in males in particular, it is possible that cortisol produced during exercise in non-exposed males (Zelnik and Goldspink, 1981) obscured any increases in cortisol in alarm-treated males. We also concede that we did not measure the extraction efficiency of our protocol, so it is possible that the quantities extracted were insufficient to reveal meaningful differences.

Surprisingly, the swim distance response of control offspring to alarm substance was markedly weaker than the responses observed in experimentally naïve animals in the preliminary experiment. This may be due either to the offspring being younger than the naïve animals, or it may be due to experimental or

rearing conditions. For instance, animals were housed individually for substantial parts of the experiment, but given that guppies are social animals, this social isolation may be regarded as a stressor (Shams, Chatterjee and Gerlai, 2015; Shams *et al.*, 2017). Because the linking of broods to individual mothers necessitated the individual housing of parents, and offspring were initially housed individually to control for population density effects, it is possible that social isolation may have influenced maternal and / or offspring physiology. It is also possible that stress imposed daily handling of both control and GPS mothers, although mild, may have been sufficient to induce changes in the offspring (Eaton *et al.*, 2015).

Further insight could be gained by evaluating foraging behaviour in young guppy offspring to assess the extent to which differences in this may underpin early growth retardation in GPS offspring, as well as the extent to which foraging ability correlates with gestation length in the presence and absence of gestational stress. As developing embryos may also have been exposed to alarm substance via the mother and would likely also have been exposed to altered maternal physiology in utero as a consequence of maternal stress, it remains unclear as to whether early alterations to developmental programming contributed to the observed effect on offspring growth. Measuring levels of cortisol, other metabolic alterations, or relevant gene expression changes in prenatal embryos would help to assess whether embryos can be altered by gestational stress via direct influences of maternal physiology. Molecular resources which are becoming increasingly available to researchers, such as the guppy transcriptome (Fraser *et al.*, 2011) would be invaluable in such studies. However, given the observed plasticity in brood retention time, guppies likely present a poor model for studying such direct influences. Conversely, given the ability of guppies to control gestation length in response to alarm substance, this phenomenon may offer a useful model for investigating the effect of gestation length on subsequent offspring growth.

Concluding remarks

Although previous studies have demonstrated the effect of predation stress on maternal reproduction and examined possible influences on early offspring behaviour (Evans, Gasparini and Pilastro, 2007),

ours is the first study to have examined long-term effects of different forms of gestational stress on the offspring of guppies. Subsequently, we have shown that gestational stress in the context of predation has important consequences for early growth in guppy offspring, which appear to be driven by shortening of the gestation period. However, the evidence for an influence of gestational stress on mature offspring was limited, implying that the effects are largely transient. That similar effects on reproduction and early growth were not detected in response to dietary restriction suggests that the effects of gestational stress vary depending on the context or severity of the stress.

Chapter 3

Lessons from the father: Paternal stress ameliorates offspring stress responses in a high-throughput vertebrate model

Abstract

A growing body of evidence points to the parental environment as having an important influence on offspring phenotype. Although the potential influence of the father's environment has long been overshadowed by that of the mother, recent evidence from rodent models suggests that paternal stress leaves an imprint on the offspring via non-genetic information carried in the sperm. The zebrafish (*Danio rerio*) is an established model in vertebrate stress research, and its rapid reproduction and fast growth of the offspring offer the opportunity to probe for intergenerational effects of stress in a high-throughput vertebrate model. Here we demonstrate that when males are subjected to stress, their larval offspring exhibit blunted behavioural and endocrine stress responses. While offspring from non-stressed (control) fathers, as expected exhibited pronounced edge preference behaviour in response to alarm substance (a model biotic stressor), this response was either weakened or not shown by the offspring of chronically-stressed fathers, which similarly failed to exhibit cortisol production in response to stressors. Furthermore, we show that males require only two brief exposures to a stressor in order to transmit an altered stress response to their offspring. Our study is, to our knowledge, the first to show that alterations to offspring stress response can be transmitted by paternal stress in a non-mammalian vertebrate.

Introduction

Stress experienced in the ancestral environment is increasingly recognised as a contributor to disease risk (Ord, Fazeli and Watt, 2017). While there is strong evidence for the influence of gestational stress, little is known about the relative contribution of the paternal environment to disease risk. The nutritional and endocrine composition of the oocyte has important influences for the developing offspring (Davis and Sandman, 2010; Best, Kurrasch and Vijayan, 2017; Redfern *et al.*, 2017), but the sperm cell was once assumed to have negligible influence beyond its role in delivering a haploid genome. However, recent findings suggest that the spermatozoon carries non-genetic information to the offspring, which can mediate environmental influences on offspring development. For instance, male mice exposed to chronic stress in early life sire offspring with reduced anxiety-like behaviours and glucose production (Gapp *et al.*, 2014), while those stressed during either adolescence or adulthood sire offspring with reduced corticosterone levels in response to stress (Rodgers *et al.*, 2013). Such findings are associated with differential noncoding RNA (specifically microRNA) expression in sperm, and, remarkably, the effects of paternal stress can be recapitulated by injecting total RNA from the sperm of stressed fathers into normal zygotes. Rodgers et al. (2015) further demonstrated that the effects of paternal stress could be recapitulated by injecting a subset of only nine paternal stress-associated microRNAs (miRNAs).

The zebrafish has long justified its place in the pantheon of vertebrate models in translational research, particularly given its short life cycle, ease of manipulation, and genetic similarity to humans. Core components of stress physiology are highly conserved between mammals and fish, for instance both utilise cortisol as their principle glucocorticoid hormone, and thus the zebrafish is also an attractive model for vertebrate stress responses (Barton, 2002). Its rapid development time (from fertilised egg to free-swimming larva in just five days) allows for rapid evaluation of the effect of developmental influences on behavioural phenotypes, and larval behaviour offers a simple and inexpensive toolset for study of stress axis function (Kalueff *et al.*, 2013). One such behaviour, thigmotaxis (edge-preference), is already an established index of anxiety-like behaviour in mammalian models (Simon, Dupuis and Costentin, 1994) and has also been demonstrated in adult zebrafish (Grossman *et al.*, 2010). In larval zebrafish, thigmotaxis has been demonstrated in both individuals and in groups, along with its

propensity for control by anxiogenic and anxiolytic drugs (Schnörr *et al.*, 2011; Lundegaard *et al.*, 2015). Its simplicity and ease of measurement renders it one of the most promising behavioural phenotypes for studying stress axis function and dysfunction in zebrafish. However, thigmotaxis elicited by anxiogenic drugs requires substantial time for these drugs to take effect. In the present study, we show that group thigmotaxis behaviour can be induced rapidly by exposure to alarm substance – a warning signal derived from the skin of adult conspecifics (Stensmyr and Maderspacher, 2012). The stimulus has previously been shown to induce pronounced behavioural (Speedie and Gerlai, 2008) and endocrine responses in zebrafish (Eachus *et al.*, 2017) and similar species (Mathuru, 2016).

Despite their established utility as models in stress research, the effects of paternal stress have not been studied in zebrafish. However, they offer a unique system in which to study the effects of paternal stress, in that the duration of spermatogenesis in adult zebrafish is approximately six days (from the onset of meiosis until the appearance of mature sperm in the efferent ducts), as established by published histological and stereological investigations (Leal *et al.*, 2009). Possible sperm-mediated paternal effects have been previously demonstrated in zebrafish in response to increased sperm competition, which affects embryo survival and hatching (Zajitschek *et al.*, 2014), and in response to paternal social status has been demonstrated to influence larval offspring activity (Zajitschek *et al.*, 2017). While such effects may be driven by genetic processes (e.g. sperm selection, genetic influences on social status), epigenetic factors carried in the sperm have been suggested to contribute. MicroRNAs, which may constitute a plausible language of germ line information transfer, are abundant in teleost fish (Babiak, 2014), and have important functions in early zebrafish development (Wienholds *et al.*, 2005). It is therefore possible that paternal miRNA delivered in sperm could influence developmental outcomes in teleosts, as has been demonstrated in mammals. We therefore hypothesised that paternal stress could induce measurable influences on relevant phenotypes in the offspring of zebrafish.

Herein, we show that behavioural and physiological responses to model stressors such as alarm substance are blunted in progeny sired by males exposed to elevated stress, supporting our hypothesis and suggesting that paternal environmental inheritance may be a conserved phenomenon across the vertebrate subphylum.

Results and discussion

Offspring of chronically stressed male zebrafish show suppressed responses to model stressors

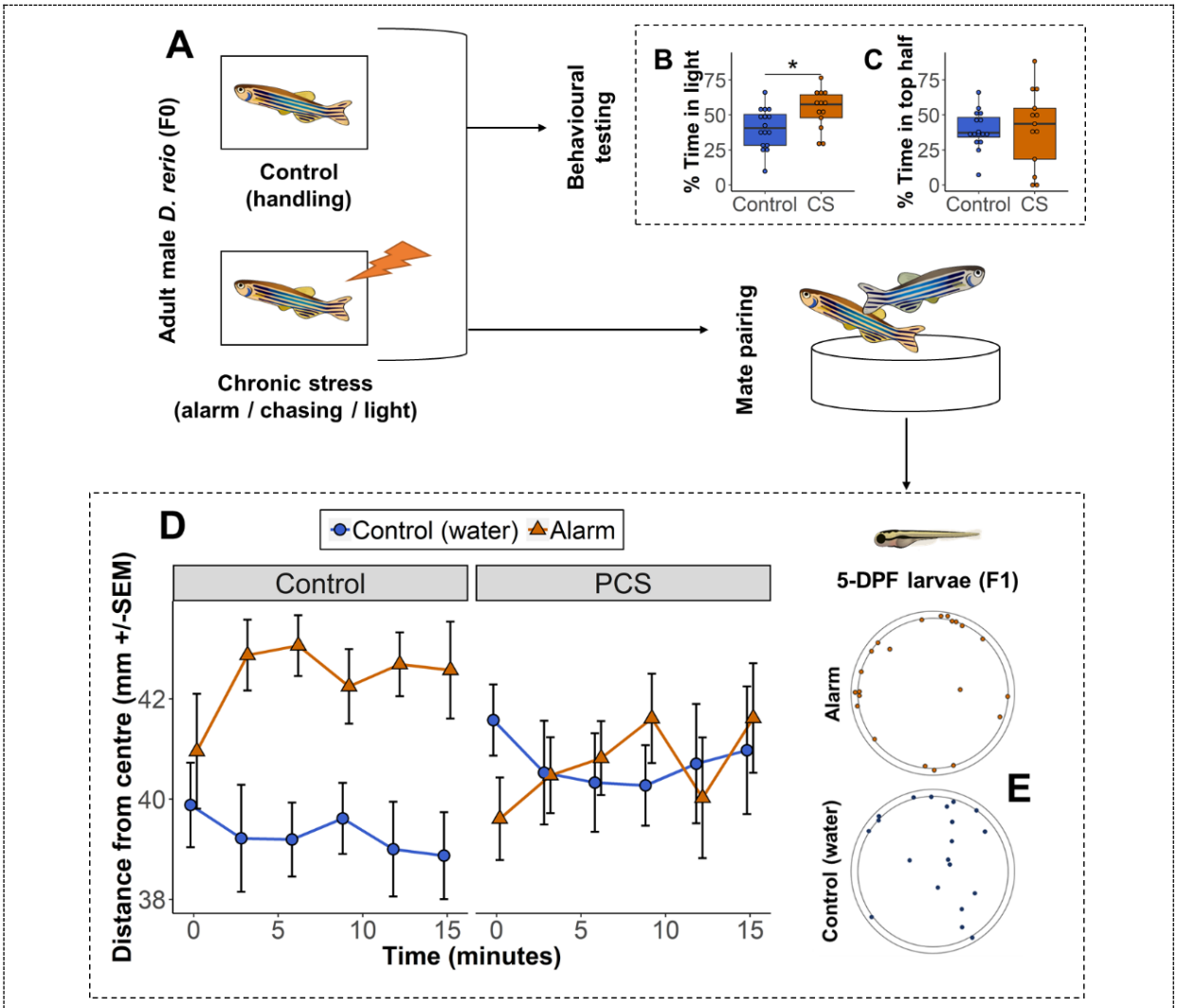

Figure 1. Visual summary of Experiment 1. (A) Adult male zebrafish exposed to a 14-day chronic stress (CS) or handling only (control). After 14 days, animals were evaluated for anxiety-like behaviour in the light / dark preference test (B) and novel tank diving test (C). (D) 5-DPF offspring were derived from control and chronic stress fathers (Paternal Chronic Stress, PCS), and thigmotaxis (edge preference) was measured over 15 minutes of alarm (orange triangles) or control (water, blue circles) exposure as the mean distance (mm) of approx. 21 animals from the centre of the dish at a given timepoint. Exposures commenced immediately prior to time '0'. Responses are presented separately for control and PCS offspring for ease of interpretation. (E) Representative maps of larval coordinates in 9cm petri dishes under alarm and control conditions after 15 mins of exposure.

Initially, we aimed to determine whether paternal stress could influence a simple behavioural stress response in the F1 progeny (Experiment 1). To this end, we exposed adult male zebrafish to a chronic stress regime comprising exposure to one of three randomly-allocated stressors per day for 14 days prior to mating to establish paternal chronic stress (PCS), alongside handled control animals (**Fig. 1A**). When tested using the light / dark preference test (**Fig. 1B**), in which animals typically favour darkness over light, chronically-stressed males spent 13.17±5.34 percent more time in the light than controls (T-test of mixed effects model, $T^{25.48} = 2.47$, $p = 0.021$). Such blunted aversion to light may be indicative of reduced stress responsivity. Control and chronic stress males did not differ in top-seeking behaviour (inverse anxiety-like measure) in the novel tank diving test ($T^{25.3} = 0.14$, $p = 0.89$), although the latter were noticeably more variable in their behaviour (**Fig. 1C**).

We subsequently derived offspring from mate pairings using chronically-stressed and control males and reared them to 5-days postfertilisation (DPF). We used thigmotaxis (edge-preference behaviour, quantified as mean distance of ~21 larvae from the central point of a petri dish) in response to conspecific-derived alarm substance as a model behavioural stress response. We compared this response between 5-days post-fertilisation (DPF) larval offspring from control and chronically stressed (PCS) males **(Fig. 1D)**. To evaluate the individual and combined effects of larval alarm treatment and PCS, we used a mixed effects model **(Table 1)** with paternal treatment, larval treatment, and time as fixed effects, and experiment batch, father ID, and dish ID as nested random effects – the last to account for repeated measurements of the same dish over time. As expected, alarm treatment induced a rapid and sustained increase in thigmotaxis in control offspring, which was highly significant ($T^{33.4} = 2.79$, $p = 0.009$). PCS offspring did not exhibit an observable response, and there was a significant interaction between PCS and larval alarm treatment ($T^{33.4} = 2.78$, $p = 0.009$). Compared to control offspring, in PCS offspring thigmotaxis appeared to be lower in response to alarm substance and higher in response to the control treatment. The results suggest that PCS induced amelioration of the behavioural stress response.

Table 1. Restricted maximum likelihood estimates and T-test values of a mixed effects model (Model 3) describing changes in thigmotaxis (mean distance of all animals from the central point of the petri dish) as a function of larval treatment, paternal stress, and time. N = 8 control pairs and 8 PCS pairs. Random effect terms are presented with the lowest level term first and higher-level terms within brackets where applicable. Batch variance is not reported as there was insufficient data for it to be estimated by the model.

Fixed effects	Estimate	S.E.	D.F.	T	p
Intercept	39.676	0.823			
Treatment (Alarm)	2.244	0.804	33.385	2.791	0.009
Paternal (PCS)	1.236	1.164	29.116	1.062	0.457
Time	-0.05	0.046	155.02	1.102	0.272
Treatment x Paternal	-3.165	1.137	33.405	2.782	0.009
Treatment x Time	0.114	0.065	155.02	1.77	0.079
Random effects	Variance	S.D.			
Dish ID: (Father ID: Batch)	1.206	1.098			
Father ID: Batch	2.832	1.683			
Residual	2.636	1.623			

In a subsequent experiment (Experiment 2), we tested offspring derived using the same experimental design as before, but measured thigmotaxis using an automated tracking software, ZebraLab® (ViewPoint Life Sciences, Inc.), which calculated the mean % of animals within 1.5 standard lengths of the dish edge during one-minute time windows. Automation of the measurement enabled us to visualise larval responses to alarm substance over time at a higher temporal resolution. In contrast to the findings of our initial experiment, both control and PCS larvae exhibited increased thigmotaxis in response to alarm cue **(Fig. 2A)**, but the response was nonetheless markedly weaker in PCS offspring, the overall mean dropping by 3.6 ± 2.5 percentage points. By the final time window, PCS offspring had reached a level of thigmotaxis closer to that of controls but appeared to reach this more slowly and with higher variability. A mixed effects model **(Table 2)** with the same parameters as those of the previous model, except with the addition of time as a cubic term (3rd order polynomial), showed that the

interaction between larval alarm treatment and PCS was not significant (T^{36} = 1.39, p = 0.17). Interestingly, there a significant interaction between paternal stress and time in the absence of alarm stress ($T^{976.1}$ = 2.54, p = 0.011), which appeared to be caused by control offspring having higher thigmotaxis at the beginning of the observation period, while PCS offspring showed a gradual increase throughout the observation period in the absence of alarm stress.

To more closely scrutinise differences between control and PCS offspring in the presence of alarm treatment, we fitted a model separately for alarm-treated offspring **(Table 3)**, which required fewer parameters and therefore allowed greater predictive power to discern effects of PCS. While this separate model did not show a significant overall reduction induced by PCS ($T^{32.4}$ = 1.43, p = 0.16), there was a highly significant interaction between paternal stress and time (T^{488} = 3.2, p = 0.002), reflecting the clear observable difference in the rate of the response between the control and PCS offspring (i.e. an abrupt response in control offspring vs. a more gradual response in PCS offspring).

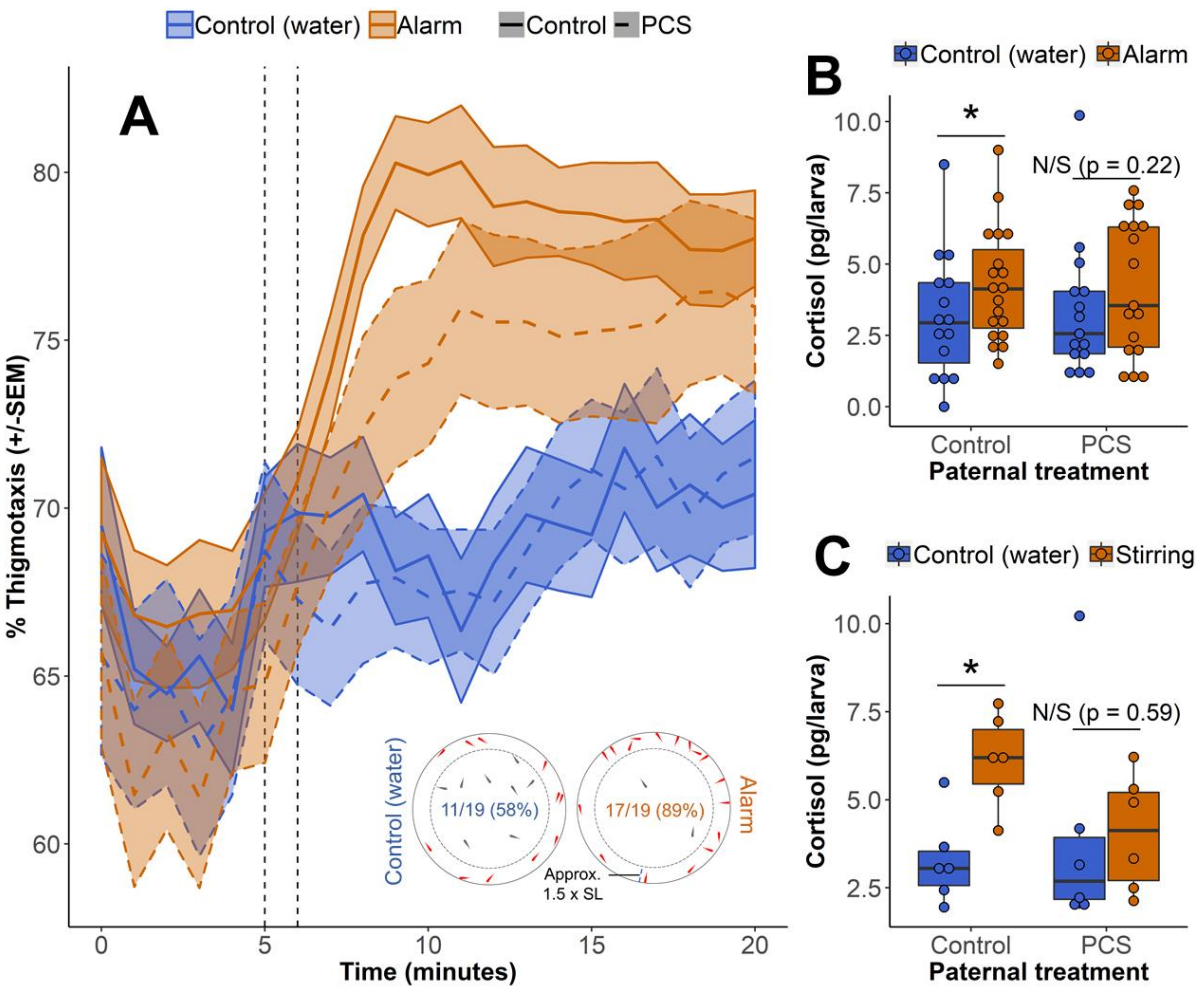

Figure 2. Visual summary of larval behavioural and physiological stress responses in Experiment 2. (A) Thigmotaxis (% of animals within 1.5 standard lengths of the dish edge) in 1-minute time bins for control (solid lines) and PCS larvae (dashed lines) exposed to alarm substance (orange) or control / water (blue). The dashed vertical lines demarcate the period during which the alarm substance was added to the dishes (between minutes 5 and 6). Inset: schematic representation of quantitation method; a larva was considered thigmotactic if it was detected within 1.5 standard lengths of the dish edge. B) Cortisol levels (expressed as picograms per larva) in the same animals following the 15-minute exposure period. C) Cortisol levels from subsets of control and PCS offspring 15-mins after exposure to a 1-min stirring stimulus, alongside equivalent subsets from the water-exposed control groups (i.e. derived from the same fathers as the stirring-treated offspring). Results are displayed with the response variable on the original response scale but were analysed on the log scale. Each point is derived from a measurement from a pool of 13-30 animals. Significance stars represent $p < 0.05$, derived from post-hoc pairwise T-tests of mixed effects models. Analyses of cortisol were carried out both with and without the prominent outliers, with significance values derived from the analyses with outliers retained.

Table 2. Restricted maximum likelihood estimates and T-test values of a mixed effects model (Model 4) describing changes in thigmotaxis (% animals in peripheral zone) as a function of larval treatment, paternal stress, and time (cubic). N = 20 control pairs and 18 PCS pairs. Nested random effect terms are presented with the lowest level term first, followed by the higher-level terms.

Fixed effects	Estimate	S.E.	D.F.	T	p
Intercept	69.753	2.013			
Treatment (Alarm)	9.013	1.678	36.015	5.371	< 0.001
Paternal (PCS)	-0.209	2.43	52.197	0.086	0.932
Time	17.686	9.504	976.061	1.861	0.063
$Time^2$	10.286	9.549	976.076	1.077	0.282
$Time^3$	-23.836	9.621	976.067	2.477	0.013
Treatment x Paternal	-3.379	2.438	35.998	1.386	0.174
Treatment x Time	-17.022	13.441	976.092	1.266	0.206
Treatment x $Time^2$	-41.505	13.505	976.114	3.073	0.002
Treatment x $Time^3$	52.854	13.606	976.101	3.885	< 0.001
Paternal x Time	34.885	13.727	976.057	2.541	0.011
Random effects	Variance	S.D.			
Dish ID: (Father ID: Batch)	26.48	5.164			
Father ID: Batch	26.589	5.156			
Batch	6.803	2.608			
Residual	23.482	4.846			

Table 3. Restricted maximum likelihood estimates and T-test values of a mixed effects model (Model 5) describing changes in thigmotaxis (% animals in peripheral zone) un the presence of alarm substance as a function of paternal stress and time (cubic). The model was fit to the same data as Model 1 but was restricted to alarm-treated animals and therefore did not include the larval treatment term. N = 20 control 18 PCS dishes. Nested random effect terms are presented with the lowest level term first, followed by higher-level terms.

Fixed effects	Estimate	S.E.	D.F.	T	p
Intercept	78.636	1.974			
Paternal (PCS)	-3.616	2.525	32.405	1.432	0.162
Time	0.482	6.726	488.021	0.072	0.943
$Time^2$	-22.061	6.758	488.026	3.265	0.001
$Time^3$	20.533	6.809	488.023	3.016	0.003
Paternal x Time	30.752	9.714	488.02	3.166	0.002
Random effects	Variance	S.D.			
Father ID: Batch	57.15	7.56			
Batch	4.212	2.052			
Residual	23.519	4.85			

Amelioration of alarm substance-induced thigmotaxis in PCS offspring implied that there was dysregulation of stress response mechanisms at the neuroendocrine level. We therefore preserved larvae from Experiment 2 following exposure to alarm substance or control treatment to test whether stress-induced cortisol production was influenced by paternal stress. We also tested a subset of control and PCS offspring (derived from the same parents) for their cortisol responses to a physical stressor (stirring), which has previously been shown to robustly induce cortisol production (Alsop and Vijayan, 2008; Jeffrey and Gilmour, 2016b), as it is possible that blunted responses to alarm substance were due to inability to detect the stimulus, rather than dysregulation of the stress response. We fitted a mixed model to each of alarm and stirring stress data separately **(Table 4)** with larval treatment and paternal stress as fixed effects, batch and father ID as nested random effects, and assay as an additional random effect.

Table 4. Restricted maximum likelihood estimates and T-test values of two mixed effects models (Models 6 and 7) describing changes in larval cortisol levels (pg / larva, log scale) as a function of larval stress treatment (alarm or stirring stress) and paternal stress. N = 66 (all alarm treatment combinations) and 24 (all stirring treatment combinations). Nested random effect terms are presented with the lowest level term first, followed by the higher-level terms. Batch variance is not reported for Model 5 as there was insufficient data for it to be estimated by the model.

Model 4: Alarm treatment	Estimate	S.E.	D.F.	T	p
Intercept	1.312	0.229			
Treatment (Alarm)	0.31	0.115	30.592	2.692	0.011
Paternal (PCS)	0.084	0.155	50.151	0.544	0.589
Treatment x Paternal	-0.162	0.164	30.14	0.986	0.332
Random effects	Variance	S.D.			
Father ID: Batch	0.073	0.271			
Batch	0.031	0.176			
Assay	0.105	0.324			
Residual	0.104	0.323			
Model 5: Stirring treatment	Estimate	S.E.	D.F.	T	p
Intercept	1.421	0.154			
Treatment (Stirring)	0.527	0.173	10	3.053	0.012
Paternal (PCS)	0.058	0.197	13.026	0.294	0.773
Treatment x Paternal	-0.43	0.244	10	1.76	0.109
Random effects	Variance	S.D.			
Father ID: Batch	0.027	0.164			
Assay	0.006	0.076			
Residual	0.089	0.299			

As expected, both stressors induced significant increases in cortisol in control offspring (alarm: $T^{30.6}$ = 2.69, P = 0.011; stirring: T^{10} = 3.05, p = 0.012) **(Fig 2, B & C)**. Although in neither case was there a significant interaction between larval stress treatment and PCS (alarm: $T^{29.4}$ = 0.99, p = 0.33; stirring:

$T^{10} = 1.76$, P = 0.11), post-hoc pairwise comparisons showed that neither stressor induced a significant increase in cortisol in PCS offspring (alarm: $T^{30.14} = 1.27$, p = 0.22; stirring: $T^{10} = 0.56$, p = 0.59). In each case, the response of PCS offspring appeared to be bimodal. Notably, cortisol under stirred conditions was lower in PCS compared to control larvae, but the difference was marginally non-significant ($T^{13} = 1.19$, p = 0.08).

Cortisol datasets were also analysed following the removal of a prominent outlier in the PCS-control (water) group which displayed considerably higher cortisol levels than any other sample. As the stirring analysis was restricted to paired samples due to the lower sample size, the corresponding stirring-treated sample was also removed. Removal of the outlier (or outlier-containing pair) largely did not affect the results. In the alarm context, the paternal x treatment interaction became less significant ($T^{26.7} = 0.66$, p = 0.51) and the effect of alarm on PCS offspring became only marginally non-significant ($T^{29.1} = 1.73$, p = 0.09). In the stirring context, removal of the outlier-containing pair similarly resulted in a less significant paternal x treatment interaction ($T^{9} = 1.57$, p = 0.15), but the effect of stirring on PCS offspring remained non-significant ($T^{9} = 1.34$, p = 0.21), and difference between control and PCS cortisol under stirred conditions became significant ($T^{13.3} = 2.9$, p = 0.01). Removal of the outlier-containing pair from the stirring dataset also negated the need to log-transform the response variable.

Although we detected influences of paternal stress on the behavioural and physiological stress responses of groups of larval offspring, we did not detect any effect of PCS on larval behaviour at the individual level when % time in dark was measured in a light / dark preference test (T-test of linear mixed model, $T^{44.63} = 0.328$, p = 0.744; **Fig. 3**), in which larvae typically exhibit preference for light over darkness (Bai *et al.*, 2016).

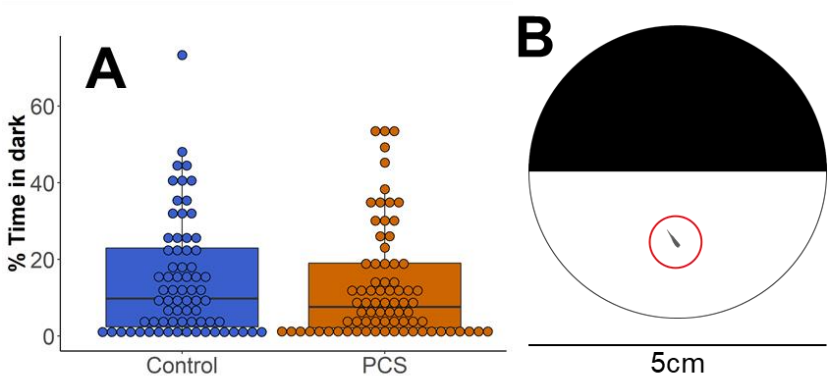

Figure 3. (A) Control and Paternal Chronic Stress (PCS) offspring in Experiment 2 did not differ in % time spent in the dark half of a 5cm light / dark testing apparatus in 7-min tests carried out at 6-7-days postfertilisation. Results represent the behavioural responses of a total of 70 control and 75 PCS larvae from 25 and 26 fathers, respectively. The apparatus (B) consisted of a 5cm petri dish with the outside of one half covered with black paper and the other half with white paper, and the dish was illuminated from below.

In Experiment 2, we also ascertained that hatching success and timing were not noticeably affected by PCS (**Fig. 4**), suggesting that the observed differences in larval stress responses were not a consequence of more general impairments to embryonic development. Rather, our results suggest that PCS offspring suffer from impairments to neuroendocrine regulation of the stress response which weaken their observable phenotypic responses. Interestingly, amelioration of behavioural and endocrine stress responses in PCS offspring is highly reminiscent of phenotypes of DISC-1 mutant zebrafish, which fail to mount stress responses and have impaired development of key regulatory neurons involved with stress response functioning (Eachus *et al.*, 2017). Furthermore, our findings are complementary to two similar paternal stress studies on rodent models, in which stress-induced corticosterone (Rodgers *et al.*, 2013) or glucose production (Gapp *et al.*, 2014) was ameliorated in offspring from stressed fathers. Importantly, this implies that aspects of the underlying mechanisms, purported to involve the delivery of noncoding RNA in sperm, are evolutionarily conserved across the vertebrate subphylum, unless they have evolved independently in mammals and fish.

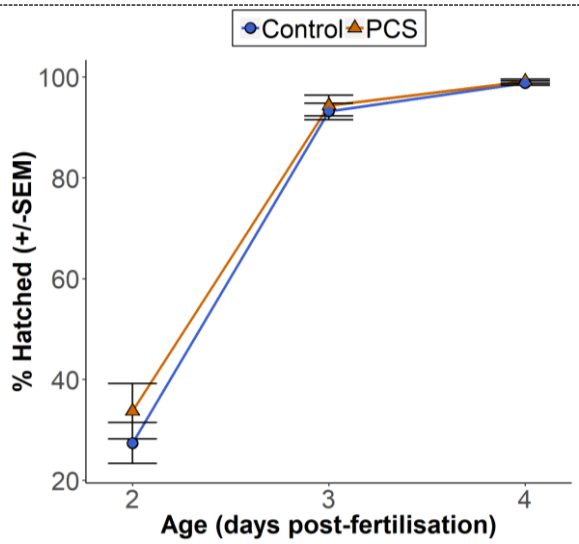

Figure 4. Percentage of embryos hatched from 2-4-DPF at 26°C for control (blue circles, N = 13) and PCS (orange triangles, N = 11) embryos in 5cm petri dishes for a subset of four batches from Experiment 2. No statistical analyses were carried out.

No prominent alterations in juvenile F1 progeny following paternal chronic stress

To evaluate whether effects of paternal stress manifested in phenotypic differences later in life, offspring from Experiment 2 were tested as juveniles (approx. 10-15 weeks post-fertilisation) using standard behavioural tests for anxiety-like behaviour **(Fig. 5)**. As well as displaying thigmotaxis, adult zebrafish have a natural tendency to avoid light and remain low in the water when presented with novelty, and therefore the open field (% movement within outer 3cm of tank as dependent variable), the light / dark preference (% time in light zone as dependent variable) and novel tank diving tests (% time in top half of tank as dependent variable) were used to measure anxiety-like behaviour (Stewart *et al.*, 2012). The open field and light / dark tests were each repeated for the same individuals over three consecutive days, while the novel tank test was carried out once for each individual. We therefore included trial (discrete categorical variable) as a fixed effect in mixed effects models **(Table 5)** when analysing light time and thigmotaxis data. There were largely no differences between control and PCS offspring detected in anxiety-like behaviour across the array of tests, with trial imposing the only overall significant effects in the case of thigmotaxis and light time. However, there was a marked trend towards increased time spent in the light zone in the light / dark preference test by PCS offspring, the mean of

which was increased by 6.96±3.9 percentage points across the three trials. Although this difference was not significant ($F^{1,20}$ = 3.18, p = 0.09), the direction of the trend is interesting as it reflects the behavioural response of F0 males to chronic stress in Experiment 1 (**Fig. 1**), and is also concordant with anxiolytic behavioural syndromes observed in rodent models of paternal stress (Gapp *et al.*, 2014). As behavioural test paradigms can be considered mildly stress-inducing, we also measured whole-body cortisol levels in offspring preserved immediately following the final test, but we found no effect of PCS on whole-body cortisol levels **(Fig. 5D)**.

Table 5. Summary of four mixed effects models (Models 9-12) for effects of paternal stress behavioural test parameters (thigmotaxis, light time, and time in top half in the novel tank test) and cortisol levels. Where applicable, trial and batch set were included as additional fixed effects. N(n) = 30(70) for light time, 30(68) for thigmotaxis and novel tank, 20(38) for cortisol, where the number outside the brackets is the number of fathers and the number inside the brackets is the number of offspring. Given the number of models represented in the table, random effect variances are not shown.

Response	Paramater	Num. DF	Den. DF	F	T	p
Thigmotaxis (% time at periphery, open field test)	Paternal (PCS)	1	28.96	0.27		0.605
	Trial (3 levels)	2	150.206	14.635		< 0.001
	Batch set (2 levels)	1	4.694	0.003		0.961
Light time (% time in light zone, light / dark preference test)	Paternal (PCS)	1	19.957	3.182		0.09
	Trial (3 levels)	2	134.901	4.264		0.016
	Batch set (2 levels)	1	5.037	2.788		0.155
Top time (% time in top half, novel tank test)	Paternal (PCS)	1	4.513	0.387		0.564
	Batch set (2 levels)	1	5.165	2.128		0.203
	Paternal x Batch set	1	4.513	2.052		0.218
Cortisol (ng / mg body weight, log scale)	Paternal (PCS)		13.042		0.333	0.745

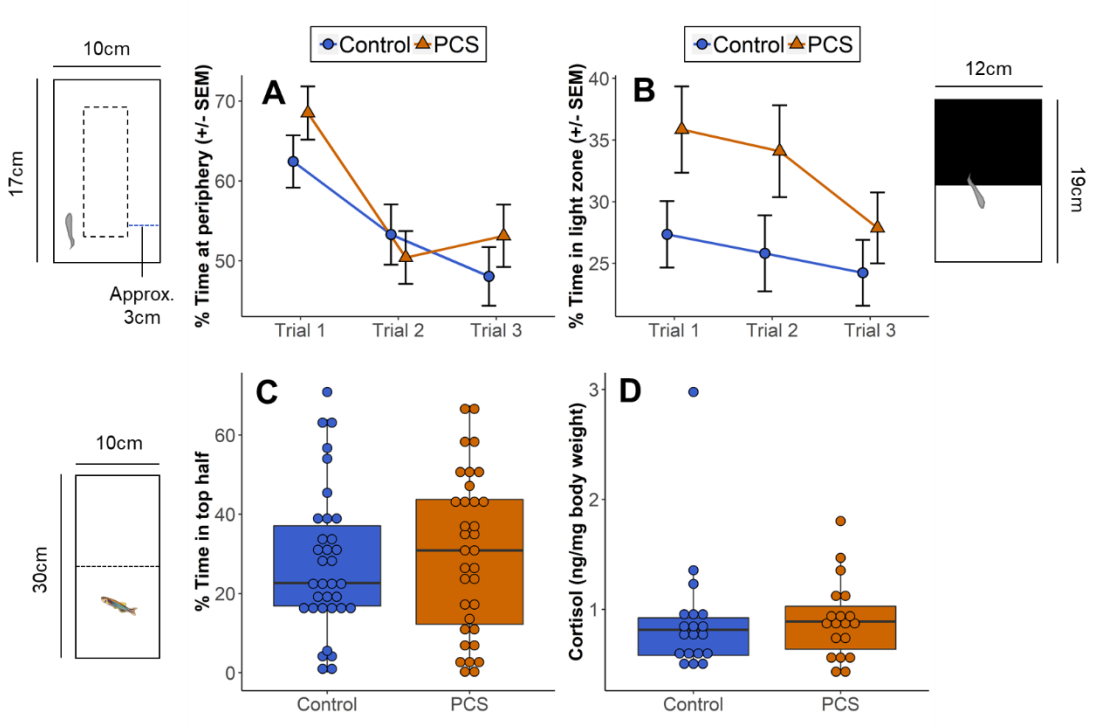

Figure 5. Anxiety-like behaviour, weight, and cortisol levels of juvenile offspring from Experiment 2. (A) Thigmotaxis (% time at the periphery of the test tank, anxiety-like measure) in the open field test and (B) light-dwelling behaviour (% time in light, inverse anxiety-like measure) in the light / dark preference test, measured from the same individuals in 5-min trials carried out on three consecutive days for control (blue circles) and PCS offspring (orange triangles) at 10-15 weeks postfertilisation. Each point is the mean for the paternal group, and bars show standard error. (C) % time in the upper half of the tank in the novel tank diving test (inverse anxiety-like measure) measured from the same individuals as in A and B but for only a single trial. Each point represents one individual. Schematic diagrams of each behavioural tests are shown alongside the relevant plots. (D) cortisol levels (ng/mg wet weight, each point the mean of two technical replicates) measured by ELISA from the same individuals.

Collectively, these results suggest that juvenile offspring traits were largely unaffected by paternal chronic stress and suggest that such influences may be subtle and context-specific, or too weak to induce long-lasting influences. This is not surprising given that a similar study using mice found no evidence of behavioural alterations in adult offspring of stressed fathers (Rodgers *et al.*, 2013). Given that developmental plasticity in the context of stress is an important driver of phenotypic variation (Schulte, 2014), long-term effects of the paternal environment may be difficult to infer in the face of the

cumulative influences of stochastic environmental variation as the organism develops. This is without mentioning the pre-existing influence of genetic variation, which may also cloud epigenetic influences on phenotype. These factors highlight the importance of effective phenotyping of embryos and larval offspring in investigations of paternal epigenetic effects.

Brief episodes of acute stress are sufficient to induce changes in subsequent offspring

While previous studies of paternal stress in other model systems have focused on prolonged (i.e. chronic) exposures in the paternal generation, the effect of shorter, acute exposures on the offspring have not been tested. In an additional experiment (Experiment 3, **Fig. 6A**), we therefore tested whether alterations to the offspring could be induced by brief acute exposures to alarm substance in the paternal generation timed around the period of spermatogenesis (approx. six days; Leal et al., 2009), as opposed to chronic stress. In addition to offspring from non-stressed controls, we tested offspring from two paternal treatment groups which received just two acute exposures to alarm substance before (pre-spermatogenesis stress; PSS: exposed to alarm on days 13 and 12 prior to mating) or during the predicted onset of the spermatogenic cycle (onset-spermatogenesis stress; OSS: exposed to alarm substance on days 6 and 5 prior to mating). We then assessed alarm-induced thigmotaxis in larval offspring using distance from centre as the thigmotaxis measurement, as in Experiment 1. Control offspring exhibited the characteristic increase in thigmotaxis, as observed in the previous two experiments. In contrast, the responses of OSS and PSS offspring, while noticeable, were clearly weaker **(Fig. 6B)**. In the case of OSS, thigmotaxis under alarm treatment had declined to the same level as control treatment by the end of the observation period. We fitted a mixed effects model **(Table 6)** with the same parameters listed for Experiment 1, with the addition of a quadratic time term (2^{nd} order polynomial). The model revealed that both paternal treatment groups influenced the response to alarm substance in some way. The interaction between paternal treatment and larval alarm treatment was significant in the case of OSS ($T^{26} = 2.27$, $p = 0.03$), while in the case of PSS was marginally non-significant ($T^{26} = 1.87$, $p = 0.07$), although the effect size was similar to OSS. We also detected a

significant three-way interaction between paternal PSS, alarm treatment, and the quadratic time term ($T^{162} = 2$, $p = 0.047$).

These data broadly mirror the observations of the effects of paternal chronic stress on offspring stress response and demonstrate that heritable effects of paternal stress are not restricted to chronic stress but can be induced even by brief exposures before or during the onset of paternal spermatogenic cycle. This suggests that developing sperm cells are far more sensitive to possible heritable epigenetic alterations than previously supposed.

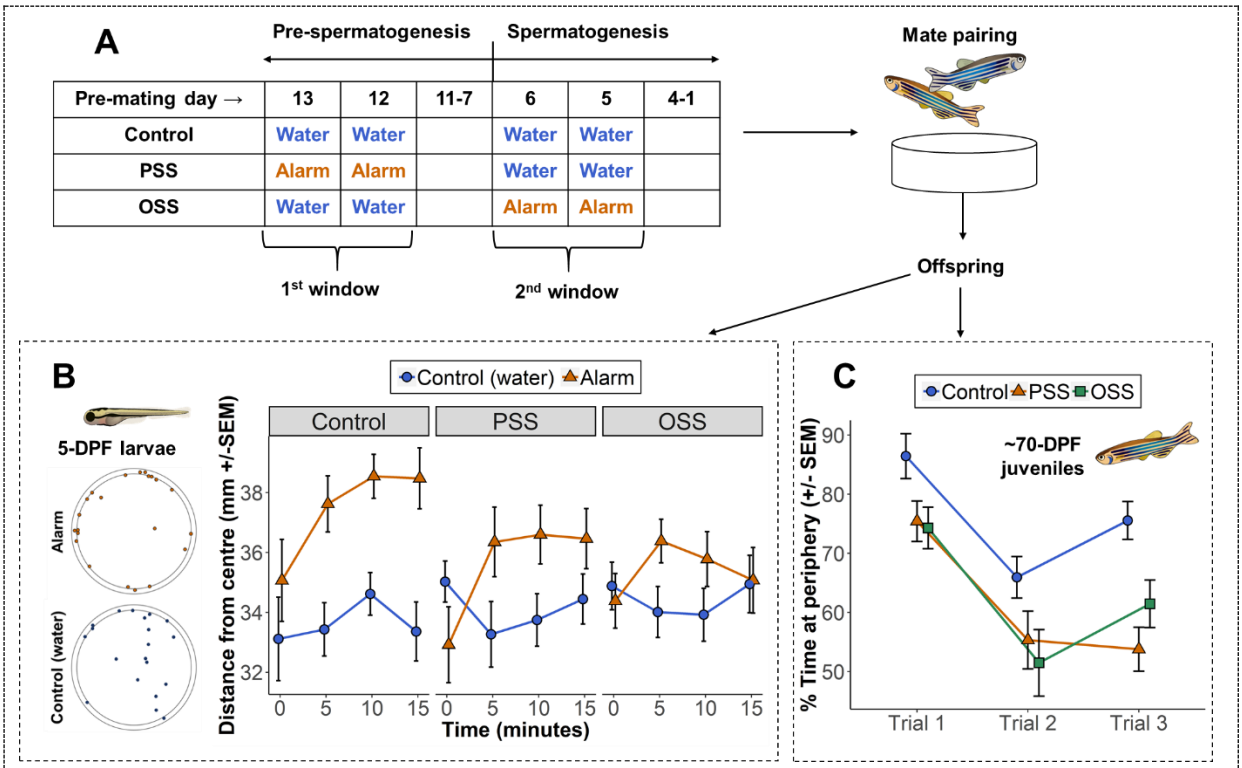

Figure 6. Visual summary of Experiment 3 (paternal acute stress). (A) table of paternal stress treatments: pre-spermatogenesis stress (PSS) fathers received 2x 20-min alarm substance exposures during the first exposure window (13 and 12 days before mating), while onset-spermatogenesis stress (OSS) fathers were exposed during the second window (6 and 5 days before mating, roughly corresponding with the onset of the spermatogenic cycle). Control animals and any animals which were not exposed during a given window were handled at the same time and administered control treatment (water). (B) In 5-DPF offspring thigmotaxis was measured over 15 minutes of alarm or control (water) exposure as the mean distance of ~21 animals from the centre of the dish. (C) Offspring from the same fathers were assessed for thigmotaxis (% time in the peripheral zone of the test tank) as juveniles (approx. 72-DPF) in open field tests repeated over three consecutive days.

Table 6. Restricted maximum likelihood estimates and test score values of a mixed effects model (Model 13) describing changes in larval group thigmotaxis as a function of larval alarm treatment, paternal stress treatment, and time (quadratic), N = 9, 10, and 10 dish pairs for Control, OSS, and PSS, respectively. Nested random effect terms are presented with the lowest level term first, followed by the higher-level terms.

Fixed effects	Estimate	S.E.	D.F.	T	p
Intercept	33.407	1.16			
Treatment (Alarm)	3.796	0.906	26	4.188	< 0.001
Paternal OSS	0.844	1.037	46.013	0.814	0.42
Paternal PSS	0.396	1.021	46.108	0.388	0.7
Time	3.283	4.851	162	0.677	0.5
$Time^2$	-5.978	4.851	162	1.232	0.22
Treatment x Paternal OSS	-2.833	1.249	26	2.268	0.032
Treatment x Paternal PSS	-2.335	1.249	26	1.869	0.073
Treatment x Time	15.691	6.86	162	2.287	0.023
Treatment x $Time^2$	-4.01	6.86	162	0.585	0.56
Paternal OSS x $Time^2$	13.182	6.686	162	1.971	0.05
Paternal PSS x $Time^2$	15.337	6.686	162	2.294	0.023
Treatment x Paternal OSS x $Time^2$	-13.484	9.456	162	1.426	0.156
Treatment x Paternal PSS x $Time^2$	-18.932	9.456	162	2.002	0.047
Random effects	Variance	S.D.			
Dish ID: (Father ID: Batch)	2.784	1.669			
Father ID: Batch	1.232	1.11			
Batch	2.362	1.537			
Residual	3.651	1.911			

Table 7. Restricted maximum likelihood estimates and test score values of a mixed effects model (Model 14) describing changes in individual juvenile thigmotaxis (% time in the outer 3cm of the test arena in 5 mins) in open field tests as a function of paternal stress treatment and trial. N(n) = 6(21), 5(18), and 7(26) for Control, OSS, and PSS, respectively, where the number outside the brackets is the number of fathers and the number inside the number of offspring. T-values derive from post-hoc tests with results averaged over the three trials. Nested random effect terms are presented with the lowest level term first, followed by the higher-level terms.

Fixed effects	Estimate	Num. DF	Den. D.F.	F	T	p
Paternal		2.000	13.819	5.751		0.015
Paternal OSS	0.273		13.58		2.939	0.011
Paternal PSS	0.21		13.15		2.681	0.019
Trial		2.000	124.478	29.461		< 0.001
Random effects	Variance	S.D.				
Fish ID: (Father ID: Batch)	49.69	7.049				
Father ID: Batch	30.58	5.53				
Batch	27.02	5.198				
Residual	244.43	15.634				

Offspring from Experiment 3 were subsequently tested as juveniles in the open field test, in which thigmotaxis was measured on three consecutive test days. Both paternal stress types induced lower thigmotaxis in each of the three trials **(Fig. 6C)**. Results of a mixed effects model **(Table 7)** showed the effect of paternal stress overall to be significant ($F^{2,13.82} = 5.75$, $p = 0.015$), and post-hoc T-tests showed that both OSS and PSS significantly affected thigmotaxis, with OSS inducing a mean reduction of 15.17±5.16 ($T^{13.58} = 2.94$, $p = 0.011$) and PSS a mean reduction of 13.23±4.93 percentage points across the three trials. Reduction in thigmotaxis represents an anxiolytic effect of paternal stress. It is interesting that we detected this in the context of acute, but not chronic paternal stress (Experiment 2), which may even be indicative of acute stress at specific timepoints having a greater heritable influence than chronic stress, although further investigation would be required to verify this. We also noticed that thigmotaxis in control offspring was higher in Experiment 3 than in Experiment 2 (**Fig 5A**). This

discrepancy is perplexing and may be the result of unaccounted-for environmental differences either in the context of the rearing environment or test conditions, given that these comprised two sets of experiments carried out at different times. It is also possible that the discrepancy reflects a heritable influence of parental housing conditions, which differed between Experiments 2 and 3 in that fathers in the latter experiment were housed in groups during the experiment (see *Experimental procedures*). One notable parallel, however is that the anxiolytic effect of paternal stress detected in Experiment 3 is concordant with the (non-significant) trend towards increased light-dwelling behaviour in PCS offspring in Experiment 2 (**Fig. 5B**), as the latter would also be generally interpreted as anxiolytic (Maximino *et al.*, 2010).

As with Experiment 2, embryo hatching did not appear to be affected by paternal stress treatments (**Fig. 7A**), and neither did post-hatching mortality between 6- and 13-DPF (**Fig. 7B**). Mixed effects models with paternal treatment and time (which was fit as a cubic term for the hatching model) as fixed effects and batch and parent as nested random effects showed no significant effect of paternal treatment on hatching ($F^{2,33.2} = 0.65$, $p = 0.53$) or post-hatching mortality ($F^{2,34.1} = 0.27$, $p = 0.76$). These results are suggestive that the subsequent behavioural alterations were not a consequence of more general developmental impairments and may be more likely to reflect specific changes in stress response physiology induced by paternal stress.

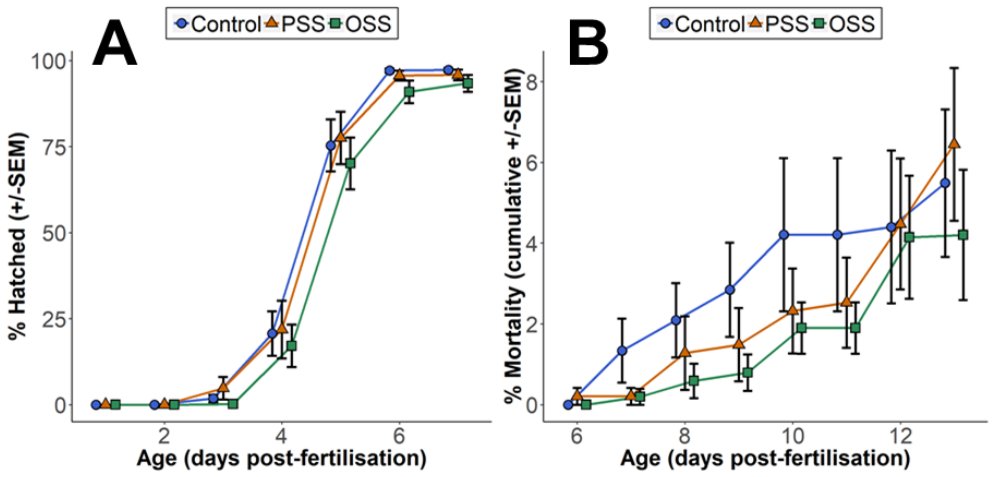

Figure 7. Life history parameters of embryonic / larval offspring in Experiment 3. (A) Percentage of embryos hatched from days 1-6 post-fertilisation for Control (blue circles, N = 13), PSS (orange triangles, N = 12) and OSS (green squares, N = 13). (B) Percentage cumulative post-hatching mortality (% of hatched embryos) from days 6-13 post-fertilisation. Embryos / larvae were reared at 25°C in open dishes of up to 40 embryos. Hatching occurred noticeably more slowly in these open dishes than in petri dishes kept at similar temperatures for Experiment 2 (**Fig. 6**), in which the majority of embryos hatched by 3- or 4-DPF. The discrepancy is likely due to differences in water temperature resultant from the presence or absence of a lid.

Overall, our data suggest that paternal stress – whether chronic or acute – has implications for offspring stress response functioning, although effects on phenotype were almost exclusively limited to the larval stage. The specific action of paternal stress was inconsistent between the three experiments, for instance in Experiment 1 the response to alarm substance was almost completely undiscernible in PCS offspring, possibly due to both blunted stress response and increased baseline thigmotaxis, while in Experiment 2 the response to alarm substance was merely weakened in a time-dependent manner. While it is possible that the two different thigmotaxis measurement methods used may have contributed to this discrepancy, it is also possible that subtle, unaccounted-for environmental differences contributed. Furthermore, we concede that we did not detect significant interactions in the case of cortisol responses, and our interpretation that cortisol responses were weakened in PCS offspring is based on the outcome of post-hoc tests which showed no significant response to the stressors in these offspring.

Nevertheless, collectively our results conform with a general pattern of weakened stress response in the F1 generation following paternal stress. This implies that environmental information can be transmitted in the paternal germ line of teleost fish, possibly in the form of alterations to the epigenetic state of sperm cells. While a similar conclusion has been implied by the findings of previous studies (Zajitschek *et al.*, 2014, 2017), ours is the first to demonstrate that exposure to stress in the paternal generation is associated with alterations in the F1 progeny. That we detected alterations in F1 progeny following either chronic stress or brief episodes of acute paternal stress would imply that germ cells are highly sensitive to epigenetic reprogramming in response to environmental events.

Possible mechanisms underlying paternal epigenetic inheritance

MicroRNAs (miRNAs) carried in sperm have previously been implicated in epigenetic inheritance of paternal stress phenotypes in rodents (Rodgers *et al.*, 2013, 2015; Gapp *et al.*, 2014). Given that we have shown similar intergenerational effects on stress response in zebrafish, and the abundance of miRNAs in teleost fish (Babiak, 2014), it is plausible that a similar mechanism is involved. A single given vertebrate miRNA has the potential to downregulate several corresponding sequences (Babiak, 2014), and thus miRNAs may constitute a powerful language to mediate environmental inheritance. In mammalian models, sperm-derived RNA has been demonstrated to become integrated into the oocyte during fertilisation, and although most of this RNA is degraded by the oocyte, some RNA species may persist until activation of the embryonic genome (Boerke, Dieleman and Gadella, 2007; Avendaño *et al.*, 2009; Yuan *et al.*, 2016). As noncoding RNAs are believed play a role in chromatin remodelling during embryogenesis (Pauli, Rinn and Schier, 2011), it is possible that sperm-derived RNA may also affect this process. Indeed, the zebrafish embryonic methylation profile may be particularly sensitive to the paternal environment, given that, in contrast to the mammalian sperm methylome, the zebrafish sperm methylome is not discarded after fertilisation, with the maternal methylome instead reprogrammed to mirror the paternal one (Jiang *et al.*, 2013; Potok *et al.*, 2013), although whether this phenomenon is widespread across the Teleostei, or merely a zebrafish idiosyncrasy, is currently unknown. Furthermore, stress has been shown to alter the sperm methylome of mice and their offspring,

despite the erasure of most gametic epigenetic marks after fertilisation in mammals (Dias and Ressler, 2013).

As we have not assessed the involvement of epigenetic factors in transmitting paternal effects, we cannot rule out the possibility of other influences, such as effects of paternal behaviour on maternal physiology during mating, environmentally-induced genomic aberrations during sperm production, or differential selection of sperm genotypes as suggested by Zajitschek *et al.* (2014). Molecular profiling of sperm DNA or RNA would therefore be a logical subsequent investigation to obtain mechanistic insights into paternal effects.

Implications for evolutionary ecology and animal welfare

Despite having long justified its place in the pantheon of vertebrate models, surprisingly little is known about the behaviour and ecology of wild zebrafish, and therefore the possible adaptive functions of the paternal epigenetic phenomena uncovered in this work are difficult to infer. Although zebrafish kept in standard laboratory conditions can spawn all year round, spawning events in wild Asian populations are purportedly confined to the monsoon season (June-September) (Spence *et al.*, 2007). However, the rapid six-day (Leal *et al.*, 2009) spermatogenic cycle allows males to potentially engage in frequent spawning events throughout the season. Therefore, if the epigenetic state of developing sperm cells is dynamically responsive to different environmental cues, a single father could contribute a high degree of epigenetic variation to their offspring within a single spawning season and indeed across multiple spawning seasons. Although some traits inherited epigenetically may prove to be maladaptive to offspring, the range of possible phenotypic variation that such a dynamic mechanism could generate would be to the overall advantage of the father in increasing the overall likelihood of transmitting his genes to subsequent generations. If phenotypic plasticity can be considered to be have a genetic basis (Scheiner and Lyman, 1991), then epigenetic inheritance may, by extension, constitute a form of genetically-encoded plasticity by which the genome itself can enjoy protection from selective pressures. One very logical hypothesis is that such intergenerational plasticity is more likely to be favoured in stable, as opposed to fluctuating environments, where the acquisition and transmission of environmental information is less risky (Hu and Barrett, 2017).

Finally, it is important to note that our findings have two major animal welfare implications. In the interest of reducing the suffering of complex animals, highly justified ethical frameworks serve to regulate the use of mammals in stress research. Because the similarity of our results to rodent models of paternal stress (Rodgers *et al.*, 2013) implies a degree of phylogenetic conservation or at least functional similarity of the underlying mechanisms, the first welfare implication is that zebrafish present a potentially attractive alternative to rodent models for the study of intergenerational effects relevant to human health. The second welfare implication is in the interest of the fish themselves, as we have shown that interesting phenotypic effects can be transmitted in response to relatively brief exposures to an ecologically-relevant stressor (alarm substance). The prolonged suffering imposed on the animal by chronic stress regimes (e.g. Piato et al. 2011) is therefore not necessary to facilitate the study of paternal epigenetic effects of stress in the zebrafish model.

Concluding remarks

We conclude that zebrafish stress response mechanisms are sensitive to intergenerational perturbation via the paternal germ line, and that the paternal environment may therefore be an important driver of developmental plasticity in teleosts. Importantly, the short duration of spermatogenesis in zebrafish facilitates intergenerational responses to environmental events of short duration, possibly serving an adaptive function. Although the underlying mechanisms are yet to be explored, we suggest that zebrafish may present an attractive, high-throughput model for further exploration of paternal epigenetic effects in the contexts of evolution, ecology, and developmental neuroscience.

Experimental procedures

Ethics statement

All animal work was carried out under a UK Home Office licence for the use of animals in scientific procedures.

Animals and housing.

Mature male London Wildtype zebrafish (aged 6-10 months) were selected from healthy stock, reared in tanks on a recirculatory system with water heated to 26°C and kept on a 12:12h light / dark cycle. Animals were fed brine shrimp and flake food twice daily.

For the chronic paternal stress experiment, adult males were housed in individual compartments (30 x 15 x 25cm) of the flow-through system for at least one week prior to the onset of the 12-14-day treatment period and for the entire duration thereof. Enrichment was provided to each fish in the form of a plastic plant. Four to five individuals (in both control and chronic stress groups) comprised one experimental batch. For Experiments 1 and 2, a total of 12 experimental batches were carried out over the course of 15 months (3 for Experiment 1 and 9 for Experiment 2), from which larval offspring behavioural, larval cortisol, juvenile behavioural, and juvenile cortisol data were variously obtained.

For Experiment 3 (paternal acute stress), animals were assigned to either one of two stress groups (see *Paternal Acute Stress*), or a handled control group. Fish in each experimental group were housed together in groups of five per compartment for at least one week prior to treatment. One batch was comprised of one group of five fish for each of control, PSS, and OSS. Four experimental batches were carried out over the course of 6 months, from which larval offspring thigmotaxis and juvenile behavioural data were obtained.

Table 8 gives the number of adult male zebrafish used in each batch of each experiment.

Alarm substance extraction.

Alarm substance was derived from mature zebrafish (indiscriminate of sex), using a modified version of a method described by Egan et al. (2009). For every 2ml of extract, 5 fish were euthanised, and 7-10

lacerations were made to the epidermis on both sides of each fish. All five fish were then placed in a single 50ml tube with 2ml water and gently shaken to allow the alarm substance to seep out. The water containing the extract was then eluted, incubated at 95°C for 16 hours, centrifuged at 10,000g for 10 mins to remove debris, and filtered through a microfilter.

Paternal chronic stress (Experiments 1 and 2)

To establish chronic stress, each animal was removed from its home tank into a separate exposure tank (17 x 11 x 12cm) once per day and administered one of three randomly allocated stressors: 1) alarm substance (200µl in 600ml water) for 20 mins, chasing with a small net in 5x 1-minute episodes over a 20-minute period, or exposure to bright light via a 10w LED tube light suspended above the tank for 20 mins. Alarm substance exposure and chasing took place in the same room as the housing tanks but with the animals obscured from view by paper or plastic sheeting, while bright light exposure took place in an adjacent room. Individuals were not visible to each other during exposures. Following alarm substance exposure, animals were moved to separate 'washing' tanks of identical dimensions to exposure tanks to remove traces of alarm substance before returning them to their home tanks. Control animals were handled in an identical fashion (e.g. moved to separate tanks of identical size to exposure tanks) but did not receive stress treatments.

Paternal acute stress (Experiment 3)

Acute stress treatment groups were exposed to alarm substance on two consecutive days, falling either 13 and 12 days before mating (pre-spermatogenesis stress, PSS), or 6 and 5 days before mating (onset-spermatogenesis, OSS), in exposure tanks (19 x 12 x 12cm) separate to their housing tanks, but in the same groups of five in which they were housed. For each exposure, 0.5ml alarm substance was administered to 2L fresh aquarium water containing the fish, following a 20-minute acclimation period. After 20 minutes of exposure, fish were moved to a second tank containing 2L fresh aquarium water for five minutes to remove traces of alarm substance before returning fish to the recirculatory system. Any groups not undergoing stress treatment at a given time point (including handled controls) were

moved to holding tanks identical to the exposure and washing tanks at the same time as stress treatment and administered the same volume of aquarium water instead of alarm substance.

Mate pairing, embryo collection and rearing.

Following treatment, male zebrafish were paired with mature females derived from healthy stock in individual compartments of a flow-through system. Marble dishes were placed in the compartments to provide a substrate conductive to courtship, and to protect embryos from cannibalism. Zebrafish typically mate at the beginning of the photoperiod, and therefore mate pairings were established in the late afternoon, for egg collection the following morning, approx. 2 hours into the photoperiod. Following removal of the dishes and removal of marbles from the dishes, eggs were rinsed, water changed, and dead eggs were removed. The following day, 1-DPF embryos from each parent were placed either in 9cm petri dishes with 30ml water (Experiments 1 and 3) or 5cm petri dishes with 15ml water (Experiment 2) in densities of 13-30 for assessment of larval stress response phenotypes. Remaining embryos were placed in 12cm plastic dishes, filled 3/4 with water, for rearing to adulthood (up to 100 per dish for Experiment 2 and up to 40 per dish for Experiment 3). Embryo dishes were kept in a temperature-controlled room (25°C, Experiments 1 and 3) or incubator (26°C, Experiment 2) under a 12:12 light cycle. Petri dishes were checked daily for hatching and mortality and dead embryos or larvae were removed. Larvae reared beyond 5-DPF were fed with commercial fry food daily from 5-DPF, and from between 7 and 15-DPF were transferred from rearing dishes to tanks (23.5 cm by 12.5 cm by 17.5 cm) on a recirculatory system with water heated to 26°C where they were housed for a further 25 days, after which siblings were transferred into larger tanks at densities of up to 15 (Experiment 2) or four (Experiment 3) and were fed commercial flake food and brine shrimp twice per day.

Larval response to alarm substance and physical stress

5-DPF offspring were tested for behavioural and physiological responses to stress. Of two sets of offspring from each parent housed in petri dishes, one was exposed to alarm substance (50µl), while the other was administered the same volume of aquarium water as a control. Exposures were carried

out whilst the petri dishes were placed on an illuminated platform to maximise contrast between fish and background (to facilitate easier observation), and video-recorded from above using Panasonic HC-X920 digital camcorders. Video recording was commenced immediately following administering of the alarm or control treatment and continued for 15 minutes, the time for cortisol to reach peak concentration in zebrafish following stress (Ramsay et al. 2009). After 15 minutes, larvae were immediately euthanised by immersion in ice water. For Experiment 2, euthanised larvae were collected into 1.5ml Eppendorf tubes and stored at -20°C until cortisol extraction.

Simultaneous to alarm exposure in Experiment 2, a subset of each of control and PCS larvae were also exposed to physical stress which was established by moderately stirring the petri dish water by hand for 1-min (~2 rotations per second), taking care to avoid physical damage to the larvae. Approx. 15 mins after stirring, larvae were collected and preserved as described above. Subsets of water-exposed control larvae (same controls as those used for alarm substance experiment) from the same fathers as stirred larvae served as controls, as stirring was initiated immediately before alarm (or water) exposure was initiated.

Larval group thigmotaxis

For Experiments 1 and 3, larval thigmotaxis was measured as the mean distance of individuals from the centre of the dish. Images of larvae were taken at times 0, 5, 10, and 15 minutes following initial exposure to alarm substance. Images were exported to ImageJ and the coordinates of all larvae were collected. Euclidean distances from each fish to the coordinates of the centre of the dish were calculated as the measure of thigmotaxis. Statistical analyses were carried out on the mean distance of larvae to the centre for each dish at each timepoint.

For Experiment 2, thigmotaxis was recorded directly from videos using automated tracking software (Viewpoint® ZebraLab) which detected and calculated the mean number of animals present in a defined central zone (approx. 1.5 standard lengths from the edge of the dish) in 1-minute time windows. This number was subtracted from the total number of animals and the percentage of animals in the peripheral zone (% thigmotaxis) for each time window was thus calculated.

Behavioural testing of juvenile offspring

To increase the likelihood that individuals tested were representative of the brood, behavioural testing was restricted to broods from which at least three offspring survived to 6-WPF. For Experiment 2, 16 control and 24 PCS broods or pools of combined broods (provided they derived from the same treatment and batch, see **Table 8**) were reared beyond 5-DPF. Of these, 10 of each met the survival criterion. Data from these 20 broods were supplemented with data from an additional 10 (5 control and 5 PCS) which were reared and tested at an earlier time (denoted as batches E1-E3 in **Table 8**). To account for this, 'batch set' was included as a fixed term in the statistical models. For Experiment 3, 6/13 control, 7/12 PSS, and 5/13 OSS broods reared beyond 5-DPF met the survival criterion. Behavioural testing was carried out when offspring were 10-15-WPF (considered 'juveniles'). At least one week prior to testing, fish were separated from siblings and kept in isolated tanks to enable repeated trials of the same offspring.

Open field trials (Experiments 2 and 3) were carried out in opaque plastic containers (17 cm x 10 cm x 7 cm) containing 500ml water. For each trial, fish were moved directly from their home tank to the testing container. The container was placed on a frosted glass platform, illuminated from below using 10 W LED tube lights to enhance contrast and maximise detection of the fish in video-tracking software (Nema et al. 2016). Fish were video-recorded for 5 minutes using a Panasonic HC-X920 digital camcorder. Water was replaced between trials to minimise the possibility of olfactory signals interfering with the behaviour of the fish. Testing always took place in the afternoon, between 12.00-15.00.

The procedure for light / dark testing was identical to open field testing except tests were carried out in tanks (19 x 12 x 12cm) covered on the outside with half white and half black paper to establish light and dark halves. Novel tank diving tests were carried out in tall glass tanks (10 x 5 x 30cm) which were illuminated from behind instead of below, and video-recorded from the front.

Experiment 2 offspring were examined in the open field (three consecutive days), the light / dark preference test (three consecutive days) and novel tank tests (single day). Experiment 3 offspring were tested only in the open field test, on three consecutive days. Immediately following the behavioural

tests (novel tank test for Experiment 2 or third open field trial for Experiment 3), fish were euthanised in 3g/l MS222, flash-frozen in liquid N_2 and stored at -80°C for subsequent cortisol analysis. Up to three offspring from each brood were tested.

Automated behavioural measurements of juvenile offspring

Video recordings were analysed using ZebraLab® automated observation software (ViewPoint Life Sciences, Inc.). For the open filed test, thigmotaxis was measured as the percentage of total time spent at the edge of the container (outer 3cm), which was calculated using the duration (in seconds) that the fish was detected within in a defined central zone. For the light / dark test and novel tank test, the total time the animal was detected in the light half or the top half was measured, respectively, and expressed as a percentage of the total test duration.

Cortisol extraction

Pools of larvae were homogenised in 2ml tubes in 120µl distilled water using a pellet mixer (Qiagen Tissue Lyser II). One ml of ethyl acetate (Sigma) was added to homogenates, tubes were vortexed for 30 seconds, incubated at room temperature for 30 mins, and centrifuged at 3000xg for 10 mins to induce phase separation. Tubes were snap-frozen on dry ice and the ethyl acetate layer was transferred to a fresh 2ml tube. The extraction was then repeated with another 1ml of ethyl acetate and the two extracts were pooled. Ethyl acetate was evaporated for 24h under a fume hood, and dried extracts were resuspended in 60µl of sample buffer (2% BSA in PBS) and stored at 4°C until ELISA (no more than one week).

Cortisol was extracted from juvenile offspring following the same protocol except that 700µl water was used for homogenisation and 500µl phosphate-buffered saline (PBS) was used to resuspend the dried extract. Excess lipid was removed from resuspended samples by adding 500µl hexane to partition the lipid fraction (Baiamonte, Brennan and Vinson, 2015). After adding hexane, tubes were vortexed and centrifuged at 6000xg for 10 mins, and the hexane layer containing the lipid was removed. Samples in PBS were then stored at 4°C until ELISA. Following a preliminary ELISA with a sample dilution series (neat, 1:2, 1:5), an appropriate dilution of 1:2 was determined for the juvenile samples.

Extraction efficiency was evaluated by spiking post-extraction homogenates with a known amount of cortisol. Larval homogenates were spiked with 50µl of 30ng/ml cortisol and extracts were resuspended in 240µl sample buffer to give a final expected concentration of 7.5ng/ml. Juvenile sample homogenates were spiked with 25µl of 500ng/ml cortisol and extracts were resuspended in 500µl PBS to give a final expected concentration of 25ng/ml. Subsequently, mean extraction efficiencies were 98% for larval samples and 145% for juvenile samples.

Cortisol ELISA

ELISA was carried out based on a previously published protocol (Yeh, Glöck and Ryu, 2013). Wells of Immulon-2 high binding 96-well plates (Thermo Scientific) were coated with rabbit anti-cortisol monoclonal antibody (1.6µg/ml) for 16 hours, washed 3x with 300µl 0.05% tween-20 in PBS, blocked with 300µl 1% BSA in PBS for 30-60 minutes, and re-washed before 50µl of sample was added with 50µl of cortisol horseradish peroxidase conjugate (1:1600 dilution). Plates were then incubated for 2 hours at room temp. on an orbital shaker and re-washed before 100µl of tetramethylbenzedene (Abcam) was added. Plates were incubated in darkness for a further 20-30 minutes at room temperature and the colour reaction was stopped by adding 100µl of 1M sulphuric acid. Absorbance at 450nm was read using a Varioskan Flash plate reader with SkanIt user interface software (Thermo Scientific).

Sample cortisol was quantitated using a standard curve generated from three replicates of each of seven cortisol concentrations (0, 0.5, 1, 5, 10, 20, and 50ng/ml). Larval samples were run across three assays and although no technical replication was carried out due to the small amount of material, mean inter- and intra-assay coefficients of variation were calculated using spike-ins (approx. 23% and 14%, respectively). Although the mean inter-assay CV was higher than normally acceptable, we accounted for inter-assay variation in the subsequent statistical analyses by incorporating assay as a random effect in the applicable models. For juveniles, a single assay was run with two technical replicates of each sample, giving an intra-assay coefficient of variation of 12%.

Prior to statistical analyses of cortisol data (both larval and juvenile), the response variable was log-transformed to normalise the distribution in the presence of outliers.

Statistical analyses

All statistical analyses were carried out in 'R' 3.4.0 (R Core Team, 2018). Because of the hierarchical structure of the experimental design and the fact that experiments were carried out in batches, we used linear mixed effects models fit by restricted maximum likelihood (REML), using the lme4 package (Bates *et al.*, 2015). In all models, 'Batch' was included as a random term. 'Father' was included as a random effect (nested within batch) when the analysis included multiple samples from the same parent (e.g. paired larval treatments), and 'Offspring ID' (or 'Dish ID') was included as a random effect (nested within Father ID) in the case of repeated trials or when observations were made at multiple timepoints. 'Assay' was included as a separate random effect in the case of cortisol analyses. Inclusion of interaction terms was determined by comparing Akaike Information Criterion (AIC) scores (using the anova() function from the stats package) between models with and without interaction terms, which were only included if they reduced the AIC score. For the group thigmotaxis models (Models 3-5 and 13) which included time as a fixed effect term, the inclusion of quadratic or cubic transformations of the time variable was similarly determined by comparing AIC scores between successive model fits (i.e. none vs. quadratic, quadratic vs. cubic), and a given transformation was included if it reduced the AIC.

Significance of fixed effect terms was evaluated using F-tests and T-tests with Kenward-Roger approximation of degrees of freedom, derived from summary tables obtained using the anova() and summary() functions from the stats package in conjunction with the lmerTest (Kuznetsova, Brockhoff and Christensen, 2017) and pbkrtest (Hakekoh and Hojsgaard, 2014) packages. Post-hoc pairwise T-tests of model terms were computed using the emmeans() function from the emmeans package (Lenth, 2019) and use Kenward-Roger approximation of degrees of freedom.

Details of the models, including model structure and sample sizes can be found in **Table 9**.

Table 8. Numbers of broods derived from mate pairings of each male zebrafish (fathers) across all three experiments. The initial number of fathers are shown along with how many of these sired broods, how many of these were tested at 5-DPF or reared for testing at the juvenile stage. Where brood numbers appear in brackets, the number outside the brackets denotes the number of broods or brood pools, while the numbers within the brackets denote the numbers of broods within each pool (1 if a single brood).

		No. fathers		No. broods obtained		No. broods tested (5-DPF)		No. broods reared		No. broods tested (juveniles)	
Experiment	Batch	Control	PCS	Control	PCS	Control	PCS	Control	PCS	Control	PCS
1	1	4	4	2	4	2	4				
	2	6	5	5	3	5	3				
	3	6	5	5	5	1	1				
2	1	5	5	5	4	5	4	3	4	2	2
	2	5	5	3	5	2	3	3	5	3	3
	3	5	5	4	3	2	2	3 (1,1,2)	3	1 (2)	1
	4	5	5	5	5	4	2	4 (1,1,1,2)	5	1 (2)	1
	5	5	5	5	5	5	4	2 (1,3)	5	2 (1,3)	2
	6	5	5	5	4	2	3	2 (1,4)	2 (1,3)	1 (4)	1 (3)
	E1	4	4	4	4			2	2	2	2
	E2	4	4	4	3			1	1	1	1
	E3	4	4	4	4			2	2	2	2

Experiment	Batch	No. fathers			No. broods obtained			No. broods tested (5-DPF)			No. broods reared			No. broods tested (juveniles)		
		Control	OSS	PSS	Control	OSS	PSS	Control	OSS	PSS	Control	OSS	PSS	Control	OSS	PSS
3	1	5	5	5	5	3	4	3	3	2	4	1	4	2	1	4
	2	5	5	5	4	5	5	4	2	4	4	3	4	1	1	2
	3	5	5	5	4	5	3				3	4	4	3	3	1
	4	5	5	5	3	5	4	3	5	4						

Notes: In Experiment 1, batch 3, only 2 of the 10 broods obtained were tested because of hatching failure in the other 8, which may have been due to contamination of petri dishes used for rearing embryos (the two that showed normal hatching were reared in fresh petri dishes). Embryos obtained from Experiment 2, batches E1-E3 were also subject to hatching failure in petri dishes, however it was possible to rear a small subset of broods from which additional embryos were kept in separate, larger dishes.

Table 9. Sample sizes, response variables, fixed and random effect terms, and nesting structure for each mixed effects model. Interactions are denoted by 'x'. For juvenile analyses, in which multiple individual offspring per parent were tested, the number outside the brackets is the number of fathers and the number inside the brackets the number of offspring. For F0 males in Experiment 1, treatment refers to the chronic stress treatment. However, for all other models in which the term 'Treatment' is used, this refers to larval stress treatment (e.g. alarm), while the paternal stress treatment term is referred to as 'Paternal'.

Experiment 1: F0 males			
Model / fixed effects terms	(Nested) random effects	N(n)	Response variable
1) Treatment	Batch	Control: 16 CS: 14	Light time (%)
2) Treatment	Batch	Control: 16 CS: 14	Top time (%)
Experiment 1: F1 larvae			
Model / fixed effects terms	(Nested) random effects	N(n)	Response variable
3) Treatment x Paternal x Time	(Batch / Father / Dish ID)	Control: 8 x pairs PCS: 8 x pairs	Thigmotaxis (distance to centre)
Experiment 2 – larvae			
Model / fixed effects terms	(Nested) random effects	N(n)	Response variable
4) Treatment x Paternal x Time^3	(Batch / Father ID / Dish ID)	Control: 20 x pairs PCS: 18 x pairs	Thigmotaxis (%)
5) Paternal x Time^3	(Batch / Dish ID)	Control: 20 PCS: 18	Thigmotaxis (%) - alarm only
6) Treatment x Paternal	(Batch / Father ID) + Assay	Control-Control: 15 Control-Alarm: 19 PCS-Control: 15 PCS-Alarm: 17	Cortisol (pg / larva, log scale)
7) Treatment x Paternal	(Batch / Father ID) + Assay	Control: 6x pairs PCS: 6x pairs	Cortisol (pg / larva, log scale)
8) Paternal	(Batch / Father ID)	Control: 25(70) PCS: 26(75)	Dark time (%) – 6-7-DPF

Table 9 contd.

Experiment 2 – juveniles			
Model / fixed effects terms	(Nested) random effects	N(n)	Response variable
9) Paternal + Trial + Batch set	(Batch / Father ID / Offspring ID)	Control: 15(35) PCS: 15(35)	Light time (%)
10) Paternal + Trial + Batch set	(Batch / Father ID / Offspring ID)	Control: 15(34) PCS: 15(34)	Thigmotaxis (%)
11) Paternal + Batch set	(Batch / Father ID)	Control: 15(34) PCS: 15(34)	Top time (%)
12) Paternal	(Batch / Father ID)	Control: 10(19) PCS: 10(19)	Cortisol (ng / mg bw, log scale)
Experiment 3 – larvae			
Model / fixed effects terms	(Nested) random effects	N(n)	Response variable
13) Treatment x Paternal x Time^2	(Batch / Father ID / Dish ID)	Control: 9 x pairs* OSS: 10 x pairs PSS: 10 x pairs	Larval thigmotaxis (distance to centre)
14) Paternal + Trial	(Batch / Father ID / Offspring ID)	Control: 6(21) OSS: 5(18) PSS: 7(26)	Juvenile thigmotaxis (%)
15) Paternal + Day^3	(Batch / Father ID)	Control: 13 OSS: 13 PSS: 12	Hatching (%) – 1-6-DPF
16) Paternal + Day	(Batch / Father ID)	Control: 13 OSS: 13 PSS: 12	Cumulative mortality (%) – 6-13-DPF

* 1 pair removed prior to analysis due to unusual strong negative response (possible labelling error)

Chapter 4

A density-gradient method for obtaining high-quality spermatozoal RNA from zebrafish (*Danio rerio*)

Abstract

The diversity of RNA found within sperm cells has become increasingly appreciated in recent years, and interest has grown markedly in light of evidence that spermatozoal RNA may contribute to the regulation of embryonic development and even mediate intergenerational epigenetic effects. However, isolation of sperm samples of suitable purity for analysis of such mechanisms presents no small challenge, especially from small vertebrates, such as zebrafish (*Danio rerio*). Density gradient-based methods are well-established for the selection of mammalian spermatozoa but are not in common use for fish species. Furthermore, the suitability of density-gradient centrifugation for obtaining purified RNA from fish spermatozoa has not been demonstrated. Herein, we demonstrate a method of obtaining purified zebrafish spermatozoa from testicular cell suspensions using density gradient centrifugation. Although a small contribution (~3%) of small non-sperm cells (possibly earlier-stage germ cells) remained following selection, the method effectively removed contamination of large non-sperm cells and large cell aggregates and increased the relative contribution of viable spermatozoa. Furthermore, RNA extracted from spermatozoa selected using this method was consistently devoid of prominent 18s and 28s ribosomal RNA peaks and enriched for small RNA peaks in fragment size distribution profiles, further indicating the removal of somatic contamination. An average of 45ng was derived from fully sexually mature individuals, or 56ng from the pooled material of five younger fish. We conclude that density gradient centrifugation is an effective method of obtaining enriched zebrafish spermatozoal RNA from small numbers of individuals and is thus a useful tool to aid in the study of RNA-mediated paternal epigenetic effects.

Introduction

Once the topic of heated debate or even deemed heretical, the inheritance of acquired traits has garnered legitimate attention in recent years thanks to data from several well-designed experiments (Chen, Yan and Duan, 2016). One of the most astonishing revelations has been that heritable phenotypic alterations can be transmitted via the male germ line. For instance, experimental data mostly deriving from rodent models (Rodgers *et al.*, 2013; Gapp *et al.*, 2014), but more recently from zebrafish (Ord et al, *in prep*), have shown that males exposed to elevated stress transmit dysregulated stress responses to their offspring. Thanks to the discovery of epigenetic mechanisms which provide plausible underlying mechanisms, the inheritance of environmentally-induced phenotypes or 'intergenerational plasticity' has found its way into an extended version of the evolutionary synthesis where it is compatible with neo-Darwinian evolution (Hu and Barrett, 2017). In mammals, one important mechanism underlying paternal inheritance comprises noncoding RNA carried in the sperm, as heritable phenotypes induced by paternal stress are recapitulated by injection of spermatozoal RNA from stressed males into otherwise unaltered zygotes (Gapp *et al.*, 2014, 2018; Rodgers *et al.*, 2015). In general, however, little is known regarding the mechanisms by which sperm acquire and transmit environmental information, especially in non-mammalian vertebrates.

Until relatively recently, mature spermatozoa were thought to be devoid of functional RNA, and any RNA that could be found in sperm cells was assumed to be transcriptional remnants from the preceding stages of maturation. However, recent evidence would suggest that mature spermatozoa from a variety of organisms contain both long and short noncoding RNA species, and a paradigm is emerging which purports that spermatozoal RNAs play important regulatory roles in early developmental processes (Boerke, Dieleman and Gadella, 2007; Yuan *et al.*, 2016) as well as acting as envoys of the ancestral environment (Gapp *et al.*, 2014, 2018; Rodgers *et al.*, 2015). In teleost fish, although they have been shown to possess spermatozoal RNA (Presslauer *et al.*, 2017), the potential postfertilisation roles of this RNA are hitherto confined to speculation. Interestingly, parthenogenetic embryos can be generated from zebrafish oocytes but require fertilisation by UV-treated sperm to initiate development, suggesting that sperm factors additional to the DNA are required (Jiang *et al.*, 2013). Moreover, prior to zygotic

genome activation in zebrafish embryos, a genomic methylation profile is established which mirrors the sperm methylome, and it has been speculated that small noncoding RNA delivered by the spermatozoon play a role in mediating this process (Jiang *et al.*, 2013; Potok *et al.*, 2013).

In mammals, it has recently been shown that RNA is delivered to maturing spermatozoa during their passage through the epididymis, via extracellular vesicles (Sharma *et al.*, 2018). Although teleost fish do not possess an epididymis (Boj, Chauvigné and Cerdà, 2015), RNA could be delivered to maturing spermatozoa enclosed within Sertoli cell-derived cysts or to mature spermatozoa within the efferent ducts (de França *et al.*, 2009). Given their previously demonstrated role in the intergenerational transmission of environmentally-induced phenotypes in mammals (Gapp *et al.*, 2014, 2018; Rodgers *et al.*, 2015), this work set out to establish a methodological framework to test the hypothesis that spermatozoal RNA is associated with the intergenerational effects of paternal stress observed previously in zebrafish (Ord et al, *in prep*).

Deriving mechanistic insights into spermatozoal RNA-dependent processes necessitates the ability to obtain high quality spermatozoal RNA which is both free from contaminating RNA from other cell types, and in high enough quantities for high-throughput transcriptional profiling. Due to the sparse nature of spermatozoal RNA, extreme care must be taken to minimise contamination by somatic and other non-sperm cells, the abundant RNA from which may heavily skew the results of subsequent analyses. Thus, derivation of abundant high quality spermatozoal RNA from zebrafish – a small teleost – presents an appreciable technical challenge, with contamination by non-sperm cells and spermatozoa quantity both presenting major obstacles.

Spermatozoa are typically collected from zebrafish either as ejaculated semen (milt) or following dissection of the testes and suspension in an appropriate solution, allowing the sperm to swim out into the solution (Jing *et al.*, 2009). Collection of milt, while bearing a lower potential for contamination by other cell types, results in low sperm yields given that a single ejaculate represents only a fraction of the potentially viable spermatozoa that may be present within the testes. Therefore, while this is a suitable collection method for examination of spermatozoal RNA of larger fish, such as seabream

(Guerra *et al.*, 2013), it is impractical for the much smaller zebrafish as a large number of ejaculates must be pooled to derive enough material for RNA sequencing (Presslauer *et al.*, 2017).

By contrast, deriving a cell suspension from dissected testes can result in up to a 20x higher yield of viable spermatozoa (Jing *et al.*, 2009), but carries a far greater risk of contamination by other cell types due to the high probability that other testicular cells will become dissociated from the tissue (I. Babiak, pers. comm.). Thus, far from being pure sperm cell suspensions, extracts collected from dissected testes present a constellation of sperm, other cells (somatics, immature germ cells, blood cells), and tissue fragments (Fig. 3 A-C). However, neither method can be considered completely 'clean' given that milt is also known to contain somatic cells (Vo *et al.*, 2015).

Because neither sperm collection method is both clean and productive, there is a need for a protocol which can derive large quantities of spermatozoa with low risk of contamination. In mammalian reproductive physiology, selection of spermatozoa via density gradient centrifugation is a well-established method for separation of spermatozoa from seminal fluid, for instance for *in-vitro* fertilisation purposes (Verheyen *et al.*, 1995; García-López, Larriba and del Mazo, 2017). Density gradients are established by layering successively increasing concentrations of colloidal silica particles in solution – commercially sold as Percoll or Ficoll – atop one another. As the lower layers are the densest, restrictions are posed on which cell types can enter these layers, according to their density. Thus, as a suspension containing spermatozoa is centrifuged through the gradient, the spermatozoa (which are smaller and denser than most other cell types) are able to collect in lower layers whilst larger, less dense contaminants are trapped in the higher layers. Numerous studies have documented the effectiveness of this method for selecting mammalian spermatozoa for purposes of RNA isolation (Cappallo-Obermann *et al.*, 2011). Previous work has shown that it is possible to apply density gradient centrifugation to piscine sperm including zebrafish (Imahara *et al.*, 2001; Li *et al.*, 2010), but the suitability of the method for piscine spermatozoal RNA extraction has hitherto not been evaluated.

Herein, we demonstrate that a Percoll density gradient centrifugation method is effective at separating zebrafish sperm from testicular cell suspensions, and that high-quality RNA can be extracted from Percoll-selected spermatozoal samples, although final RNA yields were variable. To our knowledge,

ours is the first study to demonstrate extraction of clean piscine spermatozoal RNA following selection using a density-gradient centrifugation method.

Materials and methods

Spermatozoa selection method

A number of Percoll concentration combinations were tested preliminarily before the use of a 40:80 gradient was decided. Centrifugation on 40% Percoll alone removed large cell aggregates but retained an unsatisfactory amount of small and large non-sperm cells (possibly spermatogonia).

Prior to dissection of testes, adult male Danio rerio (approx. 6 or 10 months old) were euthanised in MS-222 and placed on ice. Testes were dissected from each male and placed in 200µl ice-cold Hank's Balanced Salt Solution (HBSS), followed by gentle pipetting in and out of a cut-down P200 pipet tip, to derive a cell suspension consisting of spermatozoa and other testicular cells. Standard Isotonic Percoll was prepared with a 10:1 ratio of Percoll (GE healthcare) in 10x HEPES buffer, and subsequent dilutions of 80% and 40% were prepared with 1x HBSS. 250-300µl of 40% Percoll was carefully layered atop an equal volume of 80% Percoll, using a P10 pipette to transfer at least the first 50µl. The testicular cell suspension was smoothly layered atop the 40:80 Percoll gradient, which was then centrifuged at 900xg for 40 minutes at 4°C, with soft rotor acceleration to avoid disrupting the layers. Spermatozoa formed a whitish pellet or precipitate in the 80% phase, above which the supernatant was carefully discarded. 200µl HBSS was added to re-disperse the precipitate, which was then transferred to a fresh 1.5ml Eppendorf tube with another 200µl HBSS. Sperm were pelleted by centrifugation for 10 minutes at 1000xg.

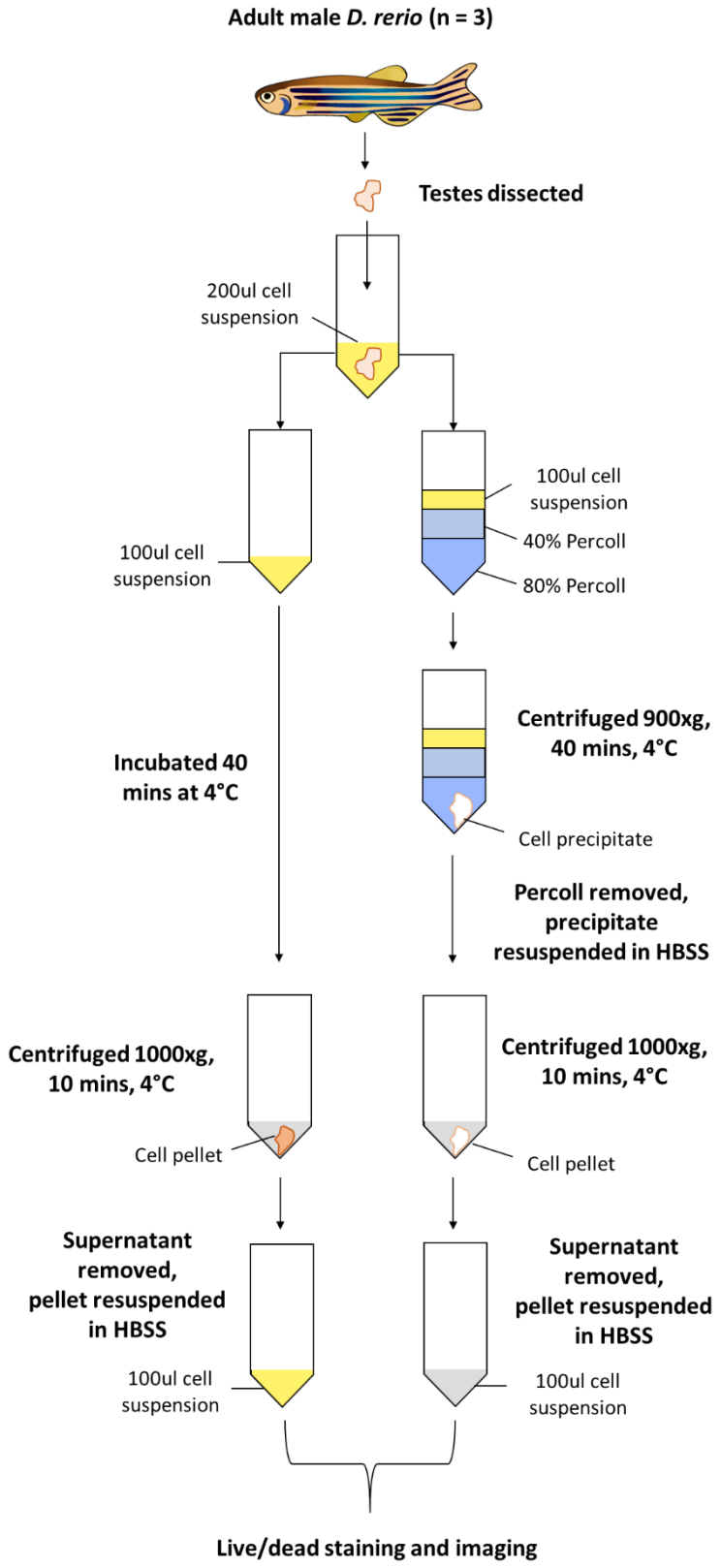

Figure 1. Schematic of experimental design. 200µl cell suspensions derived by stirring disrupting zebrafish testes in HBSS were divided into 2x 100µl fractions: one fraction was centrifuged through a 40:80 Percoll gradient, while the other was incubated at 4°C. Spermatozoa formed a white precipitate in the 80% layer following centrifugation. To remove excess Percoll, spermatozoa precipitates were washed with HBSS and pelleted by centrifugation before final resuspension in fresh HBSS. Non-selected fractions were also pelleted and resuspended to control for the effect of pelleting.

Visual evaluation of extract composition and sperm yield estimates

For microscopy-based evaluation of the Percoll-selection method, 200µl cell suspensions obtained from dissected testes of three adult *D. rerio* were each divided into 2x 100µl subsamples, of which one was subjected to Percoll-selection and the other was incubated on ice during the centrifugation period (Figure 1). Because selected samples require pelleting to remove residual Percoll, both subsamples were centrifuged and re-suspended in 100µl ice-cold HBSS prior to observation.

To distinguish between live and dead sperm cells, extracts were stained with Calcein-AM and ethidium homodimer-1 (1µl each in 50µl extract). Calcein-AM labels intact, viable cells and emits green fluorescence, while ethidium homodimer-1 labels dead or damaged cells and emits red fluorescence. 15µl of stained extract was pipetted onto a microscope slide, covered with a 24mm^2 coverslip and observed under 40x magnification using an Olympus BX-51 epifluorescence microscope. At each of five evenly-distributed sampling locations (Figure 2), three images were taken under bright-field, green fluorescence, and red fluorescence respectively. Composite images were generated using ImageJ (Rasband 1997) by merging each of the three image types for each of the sampling locations. Images were scored for the presence of various object types (see *Classification of object types for assessment of extract composition*). For each sample, an estimate of total sperm yield was calculated according to the following formula:

$$\mu_{ssi} \text{ X } S_a \text{ X } S_v$$

Where μ_{ssi} is the mean sperm count in a single image, s_a is the area scaling factor (area of coverslip divided by area of single image) and s_v is the volume scaling factor (initial extract volume divided by the volume of liquid pipetted onto the slide.

Additional images were taken under bright-field at 100x magnification to facilitate closer scrutiny of some structures.

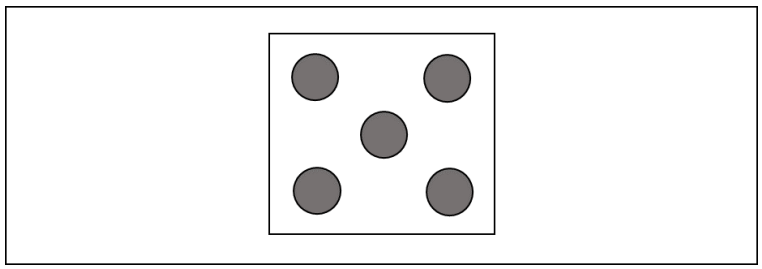

Figure 2. Representative sampling locations on 24 mm^2 coverslip at which images of testicular cell extracts were taken at 40x magnification.

Classification of object types for assessment of extract composition

Spermatozoa were identified as small round cells with a single flagellum. As several cells appeared to have lost their flagella, small round cells which could be identified as sperm heads were counted as spermatozoa. Non-sperm cells not considerably larger than spermatozoa in size were considered likely to be earlier stage germ cells (spermatids or spermatogonia) and therefore less severe contaminants than larger non-sperm cells (which are more likely to be somatic cells). Therefore, isolated non-sperm cells were divided into 'small' or 'large' categories and were placed in the latter class if they were at least three times the diameter of a spermatozoon. Large cells that appeared to be in the process of cytokinesis were counted as a single cell. Cell aggregates were divided into two categories: Type-1 aggregates consisted of six or fewer cells and appeared to contain only immature spermatozoa or spermatids; aggregates were classed as Type-2 if they contained more than six cells or if they contained large non-sperm cells, and therefore considered a more severe class of contaminating object than Type-1 aggregates.

RNA extraction

RNA was extracted either from spermatozoa pellets derived from Percoll-selected testicular cell suspensions of individual 12-month old fish (n = 13), spermatozoa pellets derived from Percoll-selected pooled testicular cell suspensions of five 6-month old fish (n = 6), or pooled lysates of individual

spermatozoa pellets derived from Percoll-selected testicular cell suspensions of three 6-month old fish (n = 2). See Table 1 for complete details of RNA samples.

The Tri-reagent system (Sigma) was chosen as it extracts total RNA including short fragments, which we anticipated to be highly enriched in the Percoll-selected sperm samples. Following purification, spermatozoa pellets were reconstituted in 200µl (for individual samples) or 700µl (for pooled samples) Tri-Reagent and mixed until complete dissolution at room temperature. Lysates were stored at -80°C until RNA extraction. RNA was extracted from lysates following the Tri-reagent protocol, with modifications. Following chloroform-induced phase separation, the aqueous phase containing RNA was mixed with 2-propanol (Sigma), and samples were incubated overnight at -20°C with 0.5µl RNA-grade glycogen (Thermo Scientific) to aid precipitation and improve visibility of the RNA pellet. Samples were then centrifuged at 14,000xg for 30 minutes at 4°C, and pellets were washed twice with ice cold 80% ethanol. Finally, pellets were air-dried for 10-15 mins and reconstituted in 12µl DEPC-treated water.

RNA quantity and quality were assessed using the Qubit fluorometer and Agilent Bioanalyser (pico chip) instruments, respectively. In many cases, RNA concentration was too low for quantitation at 1µl input using the standard Qubit protocol, and therefore a modified protocol was employed (Li *et al.*, 2015). Briefly, sample tubes were spiked with 5ng ribosomal RNA (standard 2 of the Qubit assay kit) to raise the input quantity to the minimum detection limit, thus allowing them to be quantitated by subtracting the difference in concentration from the reading derived from the spike-in alone. Because of the lack of intact ribosomal RNA in mature sperm, RNA integrity could not be judged directly from spermatozoal RNA samples according to the rRNA peaks and RNA integrity number (RIN) values provided by the Agilent Bioanalyser. Therefore, RNA was extracted simultaneously from testicular cell fractions which were not subjected to Percoll-selection. 18s and 28s rRNA peaks in these samples were used to assess the efficacy RNA isolation protocol, and thus indirectly judge the quality of extracted spermatozoal RNA.

Statistical analyses

All statistical analyses were carried out in R 3.4.0 (R Core Team, 2017). Paired students' t-tests were used to compare measures of extract composition, sperm viability, and yield.

Results

Extract composition, sperm viability, and sperm yield

Overall, the relative contribution of non-sperm cells to the composition of extracts was significantly reduced by Percoll selection from ~15% to ~3% ($t(2) = 8$, $p = 0.02$) (Fig. 5a). When different non-sperm object classes were examined, Percoll selection did not significantly affect the relative contribution of small non-sperm cells or Type-1 aggregates (appearing to consist only of immature spermatozoa), but large non-sperm cells and Type-2 aggregates were effectively eliminated (Fig. 5c). Therefore, the only non-sperm cells detected in purified extracts were small and likely to have comprised immature sperm or small earlier-stage germ cells (spermatids or spermatocytes). The relative contribution of live spermatozoa to the total sample composition was significantly increased ($t(2) = 5.52$, $p = 0.03$), but not that of dead sperm ($t(2) = 3.2$, $p = 0.08$). However, the total relative contributions of live and dead sperm in non-selected samples would have been skewed by the higher abundance of non-sperm objects. Sperm viability, measured as the percentage of total spermatozoa which were stained green as opposed to red, was higher in Percoll-selected samples, with marginal significance ($t(2) = 4.1$, $p = 0.054$). Total sperm yields in Percoll-selected and non-selected extracts varied strongly between the three replicate sample pairs and tended to be lower in Percoll-selected extracts, but the difference was not significant ($t(2)= 1.9$, $p = 0.19$).

Fig. 4B shows an enlarged image of spermatozoon heads alongside a small non-sperm cell of similar size and shape to those that rarely, but occasionally appeared in Percoll-selected samples. Following visual inspection of micrographs in the published literature, it was inferred that these cells may have been either secondary spermatocytes or intermediate spermatids – transitional cells on the cusp of final differentiation into spermatids and spermatozoa (Leal *et al.*, 2009; Schulz *et al.*, 2010). Fig. 4C shows a large aggregate appearing to consist of germ cells at various stages of differentiation (mostly

spermatids), while smaller aggregates were also seen to consist exclusively of densely-packed spermatids or immature spermatozoa (Fig. 4D). Such large aggregates were found only in non-selected samples (Figures 3B, 3C, 4C, and 4D) with most aggregates in Percoll-selected samples consisting of 2-3 cells and the largest containing six cells (Figures 3E and 4D). Large isolated round cells of the type visible in Fig. 3D could not be identified owing to their nondescript appearance, but it is possible that these were spermatogonial cells – germ cells at much earlier stages of differentiation than spermatocytes or spermatids, and which are much larger (Leal *et al.*, 2009). As with large aggregates, such isolated large cells were not detected in Percoll-selected extracts.

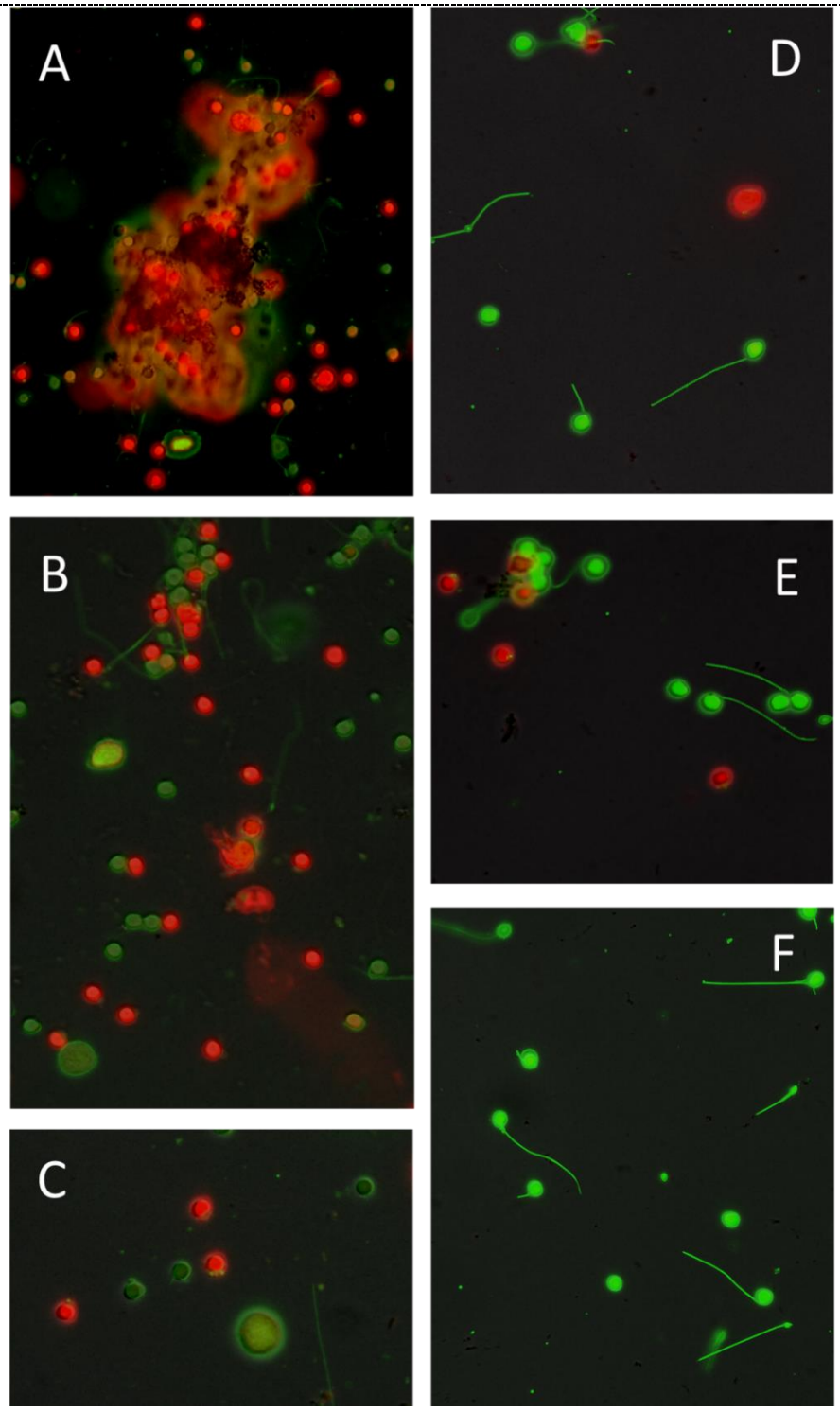

Figure 3. Composite images of non-selected (A-C) and Percoll-selected (D-F) zebrafish testicular cell extracts stained with Calcein-AM (viable cell material, green) and ethidium homodimer-1 (non-viable cell material, red), and visualised at 40x magnification. Stained objects appear brighter in images of Percoll-selected extracts as the greater diversity of objects in non-purified extracts resulted in much more intense overall fluorescence, thus exposure was lowered for capture of these images.

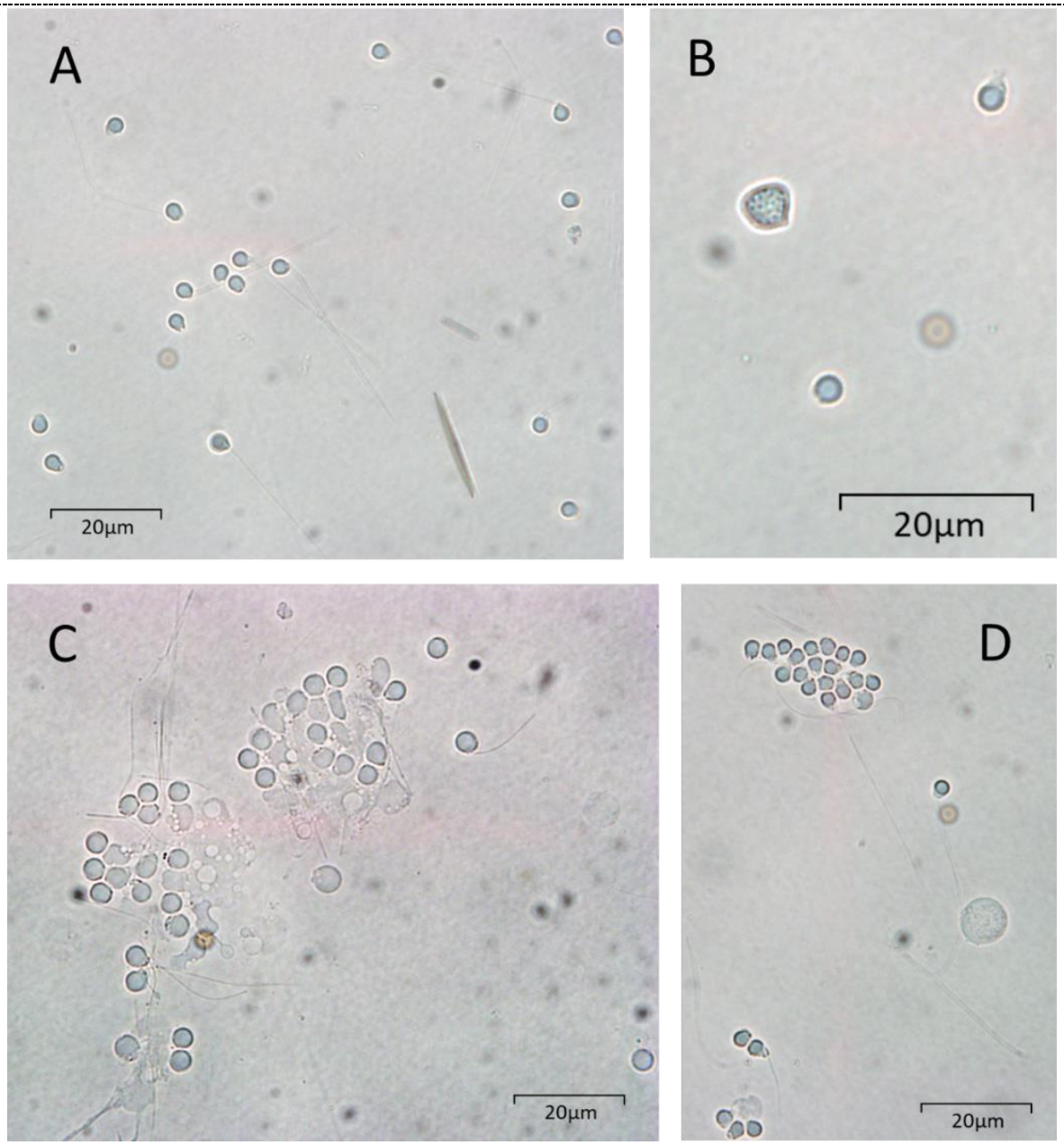

Figure 4. Bright-field images of some of the objects detected in Percoll-selected (A & B) and non-selected (C & D) zebrafish testicular cell extracts. (A) shows only mature spermatozoa or sperm heads, (B) shows two sperm heads and a small non-sperm cell, (C) shows a large (Type-2) aggregate apparently composed of germ cells at different stages of maturation, and (D) shows, in descending order, a large Type-2 aggregate apparently comprised of immature sperm, one spermatozoon, a large round non-sperm cell, one small (Type-1) aggregate appearing to consist of two immature spermatozoa, and a small Type-2 aggregate appearing to consist of three immature spermatozoa and one round non-sperm cell.

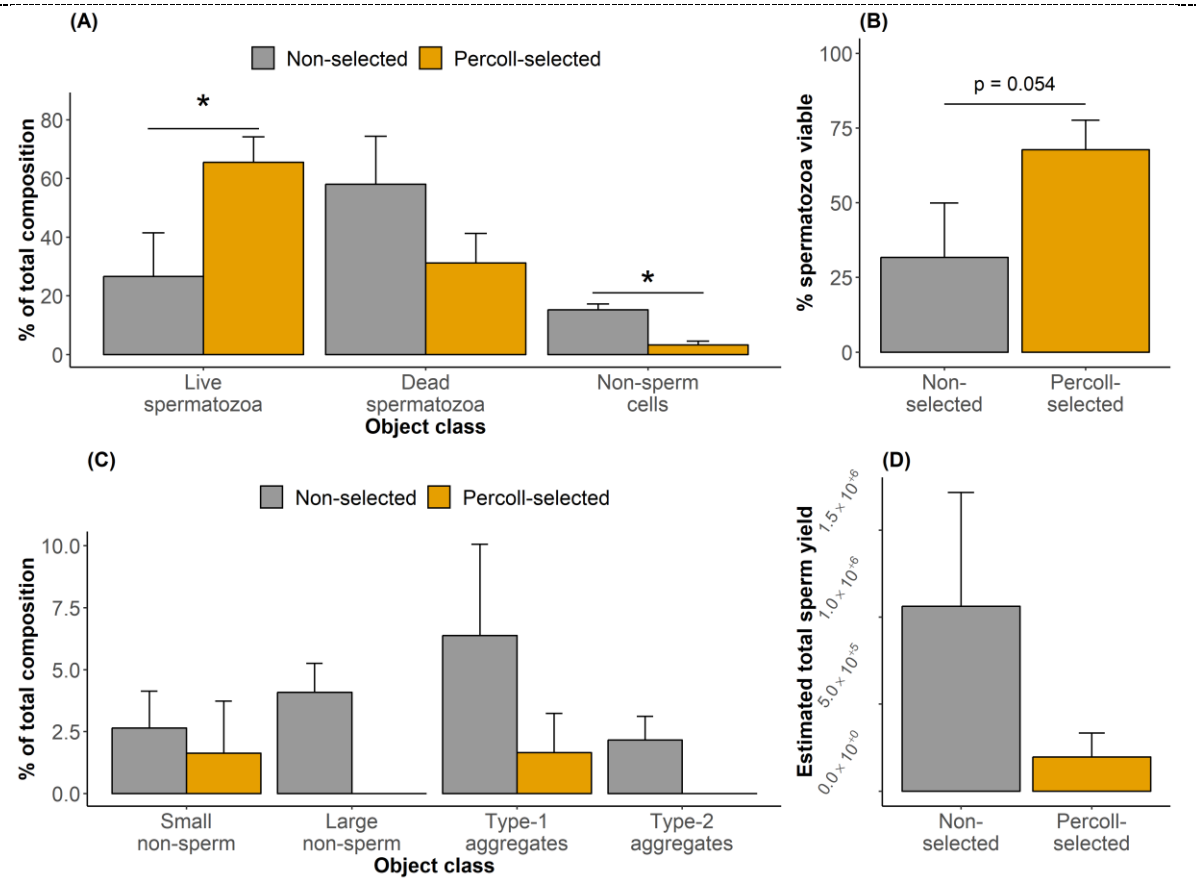

Figure 5. Metrics of cellular composition and sperm parameters of non-selected (grey bars) and Percoll-selected (yellow bars) zebrafish testicular cell extracts. (A) Relative contributions (%) of viable / live spermatozoa, non-viable / dead spermatozoa, and non-sperm objects to the total number of objects counted. (B) Sperm viability measured as the % of total sperm which were viable. (C) Relative contributions (%) of non-sperm objects to the total number of objects counted. (D) Estimated total sperm yields in 200µl of non-selected or Percoll-selected extract. N = 3 samples per category, * P < 0.05 (paired t-tests).

RNA yield from Percoll-selected spermatozoa

Spermatozoal RNA yields from all individual and pooled samples are shown in Table 1. Across all samples, the average per-animal RNA yield was 32.3ng. Yields from individual animals at full sexual maturity (12 months old) were highly variable, ranging from 9.6-110.4ng with an average yield of 45.4ng per individual (Table 1). Pooled spermatozoa from 3-5 younger fish (6-month age group) had roughly four times lower per-animal yields, ranging from 7-20ng with an average yield of 11.2ng. The mean total RNA yield from a pool of 5 young fish was 56ng. Per-animal RNA yields (averaged in the

case of pooled samples) were generally correlated with the weight of the individual or average weight of individuals in the pool (Fig. 6). However, given the two distinct sample types (pooled 6-month-old and individual 12-month-old), we did not consider it useful to statistically assess this apparent correlation.

Table 1. Spermatozoal RNA yields from all Percoll-selected sperm samples including from 12-month-old individual animals and pooled sperm samples from 6-month-old individuals. In the case of pools, the mean weights and mean per-animal yields (total yield divided by number of animals in the pool) are given.

Sample	Age Group	Sample Type	Mean animal wet weight (g)	Total RNA yield (ng)	Per animal RNA yield (ng)
1	12 months	Individual	0.42	110.4	110.4
2	12 months	Individual	0.42	28.8	28.8
3	12 months	Individual	0.38	9.6	9.6
4	12 months	Individual	0.32	50.4	50.4
5	12 months	Individual	0.37	14.4	14.4
6	12 months	Individual	0.49	43.2	43.2
7	12 months	Individual	0.47	74.4	74.4
8	12 months	Individual	0.37	36.0	36.0
9	12 months	Individual	0.36	50.4	50.4
10	12 months	Individual	0.40	69.6	69.6
11	12 months	Individual	0.35	50.4	50.4
12	12 months	Individual	0.37	9.6	9.6
13	12 months	Individual	0.46	43.2	43.2
14	6 months	Pool (n = 3)	0.34	26.4	8.8
15	6 months	Pool (n = 3)	0.31	38.4	12.8
16	6 months	Pool (n = 5)	0.28	48.0	9.6
17	6 months	Pool (n = 5)	0.28	57.6	11.5
18	6 months	Pool (n = 5)	0.27	36.0	7.2
19	6 months	Pool (n = 5)	0.29	36.0	7.2
20	6 months	Pool (n = 5)	0.28	100.6	20.1
21	6 months	Pool (n = 5)	0.30	57.6	11.5

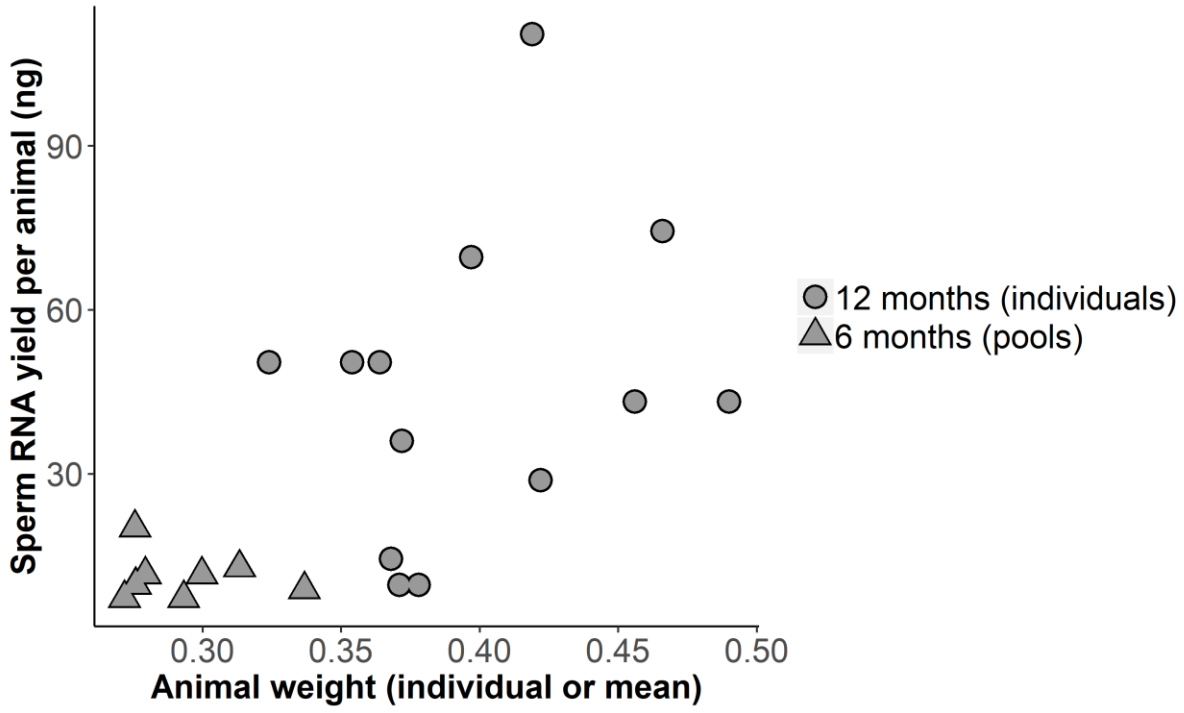

Figure 6. Spermatozoal RNA yield per animal in ng (Y axis) as a function of animal weight (X axis). RNA samples from both 12-month-old individuals and sperm pools of multiple (3-5) 6-month-old fish were included. Mean weight was therefore only applicable to pooled samples.

RNA size distribution profiles

RNA extracted from non-selected control samples displayed prominent 18s and 28s ribosomal RNA peaks (Fig 7 A and B), demonstrating effective recovery of RNA and retention of its structural integrity using the Tri-reagent-based extraction protocol. In Percoll-selected samples, the most prominent peaks were located in the small RNA region. Some samples extracted from individual fish exhibited rRNA peaks (Fig. 7 D and F), but where these appeared, they were heavily diminished compared to the small RNA peaks. RNA extracted from pooled sperm samples exhibited noticeably more consistent size distribution profiles than RNA extracted from individual sperm samples.

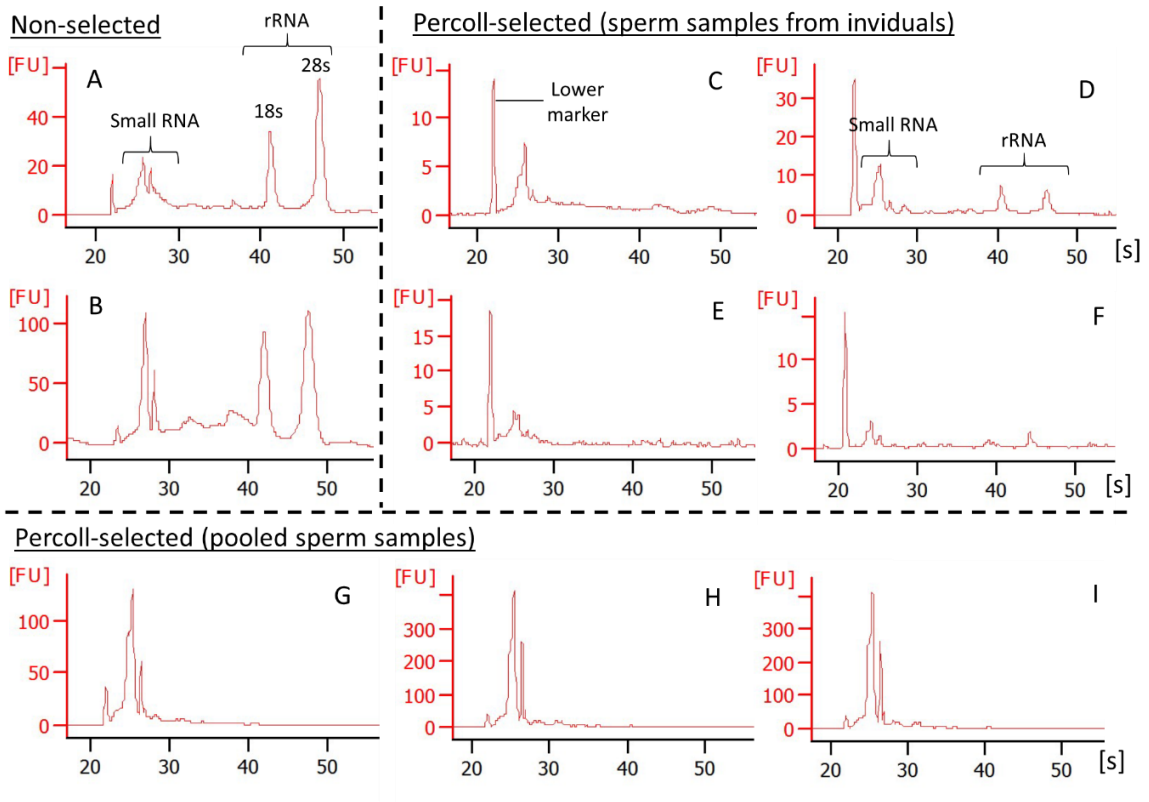

Figure 7. Representative examples of RNA size distribution profiles (electropherograms) derived from Agilent Bioanalyser Pico chips. Profiles are shown of non-selected control samples (A and B), Percoll-selected sperm samples from individual fish (C-F), and Percoll-selected pooled sperm samples (G-I). Non-selected samples exhibited prominent 18s and 28s rRNA peaks at 40-50 seconds, whilst only small RNA (20-30 seconds) was prominent in most of the Percoll-selected sperm samples. The peak observed at 22-23 seconds in all electropherograms is the lower marker (not part of the sample).

Discussion

We have demonstrated a method of zebrafish spermatozoal RNA isolation which derives material with diminished non-sperm contamination, and which is abundant enough for sequencing experiments. By examining RNA profiles from non-selected samples, we have also shown that the spermatozoal RNA is of high quality despite the expected lack of the ribosomal fractions which would otherwise be relied on for quality assessment.

We first examined the composition of selected and non-selected spermatozoal extracts to assess the degree of non-sperm contamination and the effect of Percoll selection on sperm viability. Importantly, large non-sperm cells and Type-2 aggregates were eliminated. Type-2 aggregates included very large objects of the type shown in Fig. 4C, which appeared to consist of germ cells at various stages of differentiation and likely also included somatic accessory cells. Given that germ cell differentiation is a tightly-regulated process (Schulz *et al.*, 2010), such large bodies are likely to be sites of highly active transcription and thus rich in non-spermatozoal RNA. Although the Percoll selection protocol removed these large bodies, some smaller aggregates still remained. However, given that these appeared to consist of immature spermatozoa, it is reasonable to assume that the RNA content of these cells is not substantially greater than that of mature spermatozoa, and thus would contribute minimal bias to subsequent analyses. However, this would require verification in subsequent studies. Percoll selection resulted in a more viable fraction, corroborating a previous study which applied a similar method to carp sperm and also derived a more viable fraction (Li *et al.*, 2010). Enrichment for viable spermatozoa is advantageous for the delineation of intergenerational mechanisms, as it increases the likelihood of detecting functionally important alterations which may be transmitted to offspring in viable spermatozoa.

After demonstrating that Percoll-selected samples were enriched for spermatozoa, we showed that detectable amounts of RNA can be extracted. Per-animal RNA yields appeared to positively correlate with the weight of the animal, as would be expected as larger animals tend to have larger testes and thus would have more sperm. The lower yields in younger animals reflects an established facet of zebrafish life history, in that although animals reach sexual maturity within 4 months, full reproductive capacity is not attained until 10 months. The mean per-animal RNA yield obtained using our method (32.3ng) is amenable to some types of high-throughput transcriptome-wide analyses which present the ideal means of delineating RNA-mediated mechanisms when the potential candidate molecules are not known. Although total RNA input requirements for established small RNA sequencing protocols are variable and demand, for instance up to 1µg for Illumina TruSeq, these lower limits are in continual decline as methods and technologies advance. For instance, a modification to the TruSeq protocol has enabled

sequencing using 25ng (Presslauer *et al.*, 2017), while CleanTag technology (Shore, Henderson and McCaffrey, 2018) uses chemically-modified adaptors which reduce the formation of adaptor-dimers which would otherwise be a hindrance at low inputs, thus allowing total RNA inputs as low as 10pg. With sequencing now possible using such low inputs it is possible that sequencing could be achieved using material from a single zebrafish ejaculate, and thus it may be suggested that the additional work required to Percoll-select zebrafish spermatozoa from testicular cell suspensions is already a redundant affair. However, as ejaculate collection is carried out under anaesthesia and thus requires legal permission in certain legislative areas, selection of sperm from testicular cell suspensions carries the advantage of not requiring the animal to be alive and thus not comprising a regulated procedure. Furthermore, higher yields of RNA provide a greater margin for error, as substantially more excess material would be available if sequencing experiments needed to be repeated.

The size distribution profiles of RNA extracted from Percoll-selected spermatozoa showed comparable characteristics to those of spermatozoal RNA from other organisms presented in the literature, notably the exaggerated contribution of small RNA fragments and the absence or diminished nature of the ribosomal RNA peaks (Johnson *et al.*, 2011; Kawano *et al.*, 2012; García-López, Larriba and del Mazo, 2017). Two previous mammalian studies (Cappallo-Obermann *et al.*, 2011; Georgiadis *et al.*, 2015) have demonstrated that density gradient centrifugation reduces non-sperm cell content of ejaculates and that this directly correlates with the presence of the rRNA peaks. Although we did not directly assess the relationship between non-sperm cell content and rRNA, the results obtained using our methods for fish sperm RNA corroborate these previous findings, as we found that density gradient centrifugation resulted in both reduced non-sperm cell contribution and diminished rRNA. Ribosomal peaks remained visible in some Percoll-selected samples, but never exceeded the small RNA region in height. This rRNA could have arisen from the small proportion of non-sperm cells within the samples, or from the spermatozoa themselves which may contain some residual rRNA. Another possibility is that rRNA in spermatozoal samples could arise from bacteria. However, a small contribution of bacterial RNA would not likely pose an appreciable hindrance to subsequent RNA-seq analyses as these fragments would not be mapped to the reference genome. Importantly, the prominence of the small RNA fractions in Percoll-

selected samples implies a substantial enrichment for spermatozoal RNA compared to non-selected samples, so even if rRNA peaks arise from non-sperm contaminants, their substantially reduced relative contribution to the sample makes them less likely to confound the detection of treatment-induced changes to spermatozoal RNA than if the extracts were non-selected. The greater consistency in appearance of pooled sample RNA distribution profiles compared to individuals is to be expected given that pooled samples are more representative of a population. This consistency serves to illustrate an experimental advantage of pooling spermatozoa, as detection of treatment-induced effects on RNA composition are less likely to be confounded by inter-individual variation.

One notable disadvantage of the selection protocol is that layered gradients can be tedious or laborious to establish. Furthermore, we did not assess the quantity and quality of RNA extracted from spermatozoa collected or selected by other methods. For instance, the 'panning' method utilises antibodies complementary to cell surface markers to bind either non-sperm cells or the spermatozoa themselves (Pelengaris and Moore, 1995), while a method relying on differential rates of cell sedimentation has also been applied previously to mammalian cells (Willison *et al.*, 1990). A future study could compare different methods of spermatozoa extraction and selection and their suitability for RNA isolation.

Concluding remarks

In conclusion, we have successfully demonstrated a density-gradient centrifugation protocol for zebrafish spermatozoa selection which is suitable for subsequent isolation of spermatozoal RNA. The method produced samples substantially enriched for viable spermatozoa, depleted in non-sperm contaminants, and with RNA profiles characteristic of spermatozoal RNA. Furthermore, spermatozoal RNA can be derived in quantities suitable for RNA-sequencing from fully mature individuals or from the pooled material of a small number of younger individuals. While there is scope for further optimisation, addressing of uncertainties (e.g. RNA contribution from immature sperm cells), and comparison with other methods of collection and selection, we suggest that our protocol could present a valuable asset in efforts to delineate paternal RNA-mediated intergenerational effects in teleost fish.

Chapter 5

General Discussion

"We can't any longer have the conventional understanding of genetics which everybody peddles because it is increasingly obvious that epigenetics - actually things which influence the genome's function - are much more important than we realised."

Sir Robert Winston

This project has explored effects of the parental environment on the offspring of two model teleost fish – the guppy (*Poecilia reticulata*) and the zebrafish (*Danio rerio*) – within the conceptual frameworks of stress, periconception, and epigenetics. This discussion will reflect on the work and develop new ideas across three sections. I will begin in the first section with a summary of the findings, before proceeding to highlight some of the common themes that emerged from the two different models and placing the findings within a broader set of contexts, particularly that of human disease. I will then acknowledge some of the major limitations of the work, namely the potential confounding sources of variation, as well as methodological limitations. For the remainder of the discussion, I will develop a number of new hypotheses to address questions which have arisen from the findings of Chapter 3 (comprising the most substantial body of experimental work), principally concerning the possible mechanisms underlying the paternal transmission of environmental information, and finally suggest experimental approaches with which to address these questions.

Summary of findings, common themes, and broad implications

Chapter 1 introduced concepts of stress and the potential of environmental stress to induce long-term effects on human health and cognitive function, and indeed reverberate from one generation to the next, via broadly-defined epigenetic mechanisms. Stress was defined in the framework of neuroendocrine mechanisms which are activated in response to environmental stimuli, specifically the hypothalamic-pituitary-adrenal (HPA) axis in mammals, or its equivalent in teleosts, the hypothalamic-pituitary-interrenal (HPI) axis. Animal models are invaluable tools for studying parental effects of stress and their underlying mechanisms, but while most of this work has been previously carried out in rodents, the potential utility of teleost fish in this regard had hitherto been under-explored.

Chapter 2 described offspring phenotypic consequences of maternal gestational stress in one model teleost, the live-bearing guppy (*Poecilia reticulata*). Offspring from mothers exposed to elevated stress were stunted in the early growth period and failed to exhibit a behavioural response to a model stressor in adulthood, suggestive of possible environmentally-induced epigenetic effects. However, the most pronounced effect of gestational stress was shortening of the mother's gestation period, such that offspring from stressed mothers tended to be released earlier, and likely at an earlier stage of development. Therefore, any epigenetic mechanisms in the form of maternal-embryo communication in-utero (which are of key interest in the human disease context) would likely be of diminished importance compared to mechanisms taking place in the early postnatal environment. Nevertheless, long-term alterations were observed following changes to the embryonic environment (in this case, an earlier shift from the intrauterine environment to the open water), suggesting that guppies may serve as a useful model for long-term environmental effects of stress during the critical period of embryonic development.

The zebrafish (*Danio rerio*) is a well-established model in developmental neuroscience, whose external modes of fertilisation and embryo development remove the confounding effect of variation in internal development time. This externality of development, in addition to the rapid spermatogenic cycle in the adult male, renders the zebrafish an attractive model for the study of paternal intergenerational effects of stress – phenomena previously uncovered in mammals and which appear to be mediated by

noncoding RNA carried in the sperm. Subsequently, Chapter 3 – comprising the vast majority of the work carried out for this thesis – detailed the intergenerational phenotypic consequences of paternal stress in zebrafish. Given the expected subtlety of paternal epigenetic inheritance, it was not surprising that phenotypic alterations were of a limited nature. The most dramatic and consistent phenomenon, however, was that larval offspring sired by stressed fathers failed to exhibit the profound behavioural response to alarm substance to the extent that was observed in control offspring. These findings broadly corroborate with those reported in the rodent literature (Dias and Ressler, 2013; Rodgers *et al.*, 2013; Gapp *et al.*, 2014), suggesting that aspects of the underlying mechanism are evolutionarily conserved. This work went a step beyond previous (chronic stress-oriented) rodent studies, however, in showing that intergenerational alterations can be induced in following just two brief exposures to a stressor coincident with spermatogenesis in the paternal generation.

The findings from both sets of experiments posed questions as to the mechanisms underlying the observed disruption to offspring stress response by parental stress. In the case of gestational stress in guppies, these mechanisms may comprise both pre- and post-natal influences throughout the lifespan of the progeny. By contrast, the paternal effects observed in zebrafish were enacted over a short timeframe and manifested in the progeny at a very young age, suggestive of an elegant set of mechanisms which are disrupted in the early embryo in a sperm-dependent manner. The possibility of these mechanisms existing (also as evidenced from the rodent literature) was the impetus for a planned high throughput transcriptomic analysis of zebrafish spermatozoa, for which substantial foundations were established and reported in Chapter 4. Detailing a method developed for isolation of high-quality RNA from zebrafish spermatozoa, Chapter 4 paves the way for a new set of potential experiments to explore the molecular events underpinning paternal epigenetic effects in teleosts.

Although having shone the searchlight on different model teleosts and different types of exposure, common themes emerged from the findings of these phenotype-oriented experiments. In neither experimental system were consistent parental effects detected using standard tests for anxiety-like behaviour, suggesting that parental influences did not have an appreciable influence on these traits compared to other sources of variation (genetic or environmental). However, when the offspring

themselves were tested for their response to a stressor, reduced or blunted stress responses were apparent following parental stress in both guppy and zebrafish systems (albeit not significant in the case of guppies). This commonality suggests that similar outcomes (dysregulation of stress response) can arise through different modes of perturbation in the parental environment.

Dysregulation of the stress response following gestational or paternal exposure in the teleost models is reminiscent of phenomena previously observed in rodent models, and therefore fish appear to be susceptible to intergenerational effects of stress in a similar manner to humans and other mammals (Harris and Seckl, 2011; Rodgers *et al.*, 2013). Parental effects may serve an adaptive function, for instance to 'prime' offspring for enhanced survival under stressful conditions (Donelson *et al.*, 2011; Mohan *et al.*, 2018). However, this leads to one of the most interesting observations common to both guppy and zebrafish experiments – that behavioural responses to alarm substance were weakened, not enhanced, following parental stress. This would seem to be paradoxical as in higher-predation environments reduced sensitivity to predator cues would more likely be maladaptive than adaptive.

The explanation for this phenomenon may be very complex and relate to several interconnected aspects of the animals' ecology, physiology, and anatomy. For instance, it is possible that as the level of perceived predation in the environment increases above a certain level, the 'usefulness' of the alarm response diminishes relative to the usefulness of other sensory mechanisms or other adaptive traits that are expressed constitutively. Indeed, the same idea can be applied to the stress response in general. As described in Chapter 1, activation of the stress response is costly, and thus even though it represents an adaptive mechanism, repeated activation is detrimental to fitness. Animals could avoid incurring a high fitness cost in 'high stress' environments by reducing stress responsivity. However, as they would no longer be able to rely on the stress response for their survival to the same extent, animals would need to compensate for reduced stress responsivity by diverting resources to constitutively-expressed traits which aid survival – for instance, behavioural and morphological adjustments in the context of predation stress (Arnett and Kinnison, 2016). This idea is compatible with established notions of phenotypic plasticity and could be applied to an intergenerational framework, thus providing a potential adaptive explanation for the observed findings.

However, interpretation in an ecological framework is further complicated by the laboratory context of the experiments, including the relative domestication of the animals compared to wild varieties, the lack of environmental complexity, and the use of refined alarm substance at concentrations which may not necessarily reflect exposures in the wild.

Given that prenatal (Chapter 2) and paternal stress (Chapter 3) induced alterations to offspring phenotype, the findings can be interpreted within the framework of the Developmental Origins of Health and Disease (DOHaD) hypothesis. Applying the notion of phenotypic plasticity to human health, DOHaD purports that susceptibility to disease in adulthood results from a mismatch between prenatal and postnatal conditions due to developmental changes which would otherwise be adaptive (Feil and Fraga, 2012; Soom, Fazeli and Kuran, 2013). Initially, this concept was principally studied in the context of prenatal dietary influences on the risk of developing metabolic syndromes, but has since been extended to prenatal stress which is associated with adverse neurological effects on offspring (Thayer *et al.*, 2018), possibly via adaptive mechanisms. A proposed further extension to DOHaD would accommodate paternal environmental influences (Romanus, Neven and Soubry, 2016), and therefore developmental consequences may arise from mismatch between environments encountered by sperm, developing embryos, and the developed organism.

Although phenotypic consequences of both prenatal and paternal stress were detected, significant effects on offspring phenotype were for the most part limited to early developmental stages, and the findings are therefore only partially consistent with DOHaD. In guppies (Chapter 2), the principal phenotypic effect of gestational stress was the reduction in growth rate in the first two weeks post-parturition. Although there were apparent trends towards blunted stress response in adult offspring, these effects were not significant, and no clear differences were apparent in offspring weight or their behaviour in standard behavioural tests. Similarly, in the case of chronic paternal stress in zebrafish, no behavioural or physiological alterations were detected in adult offspring (Chapter 3, Experiment 2). In only one experiment were their significant alterations detected in adult offspring in response to paternal stress, and this was in the case of paternal acute stress (Chapter 3, Experiment 3).

Incidentally, one of the predictions of DOHaD is that certain stressors may induce stronger effects on phenotype if limited to a defined time period or 'critical window' during early development. For instance, gestational low protein diet in mice has stronger deleterious effects on offspring growth and metabolism when restricted to early gestation than when imposed throughout gestation (Watkins *et al.*, 2008; Fleming *et al.*, 2012), while similar 'critical windows' have recently been elucidated in seahorse pregnancy (Otero Ferer et al, *in prep*). Such critical windows may also apply to spermatogenesis given that paternal effects of stress (Chapter 3) were not only present when stress was restricted to two brief episodes around spermatogenesis, but these targeted stress treatments also revealed an effect on adult offspring behavioural phenotype which was not present in the context of paternal chronic stress (maintained throughout spermatogenesis).

As well as showing that fish are a suitable model with which to address questions relating to DOHaD, the findings of this work have broad implications for evolutionary biology and animal welfare, as already briefly discussed in Chapter 3 and thus will not be repeated here. In addition, the findings may be important in the context of aquaculture as farmed fish species are housed in semi-natural conditions which are subject to environmental variability (Gavery and Roberts, 2017). Aquaculture is a rapidly-growing sector and a promising component of future food security, but stress and disease threaten the health of farmed fish and thus aquacultural productivity (Leung and Bates, 2012). An appreciation of environmental epigenetic effects in teleosts may therefore help to optimise rearing and breeding conditions towards production of healthier stock.

Limitations

The subtlety of parental effects imposes an inherent difficulty on efforts to study them, given that the cumulative influence of other sources of variation may mask the effects of experimental manipulation on a given phenotypic trait, such as a behavioural parameter. Underlying genetic variation and (somewhat ironically) environmental epigenetic influences may comprise much of this confounding variation. Technical and methodological constraints, however, may also have constrained the accuracy

or reliability of the results. Many of these limitations suggest valid areas for further research and even pose fascinating questions in themselves.

Genetic and epigenetic variation

Although derived from inbred lines, some degree of genetic heterogeneity can be expected in the populations of experimental fish. Indeed, inbred zebrafish strains are more genetically diverse than other commonly-used model organisms such as mice and rats (Guryev *et al.*, 2006), and population genomics analyses have shown extensive levels of germ-line variation and population substructure in within laboratory zebrafish strains (Brown *et al.*, 2012). Just as epigenetic alterations associated with key HPI axis genes (e.g. NR3C1, OGT, POMC, 11β-HSD) may affect their transcription and contribute to phenotypic variation in stress response and behaviour, classical genetic mutations within these genes or associated enhancer or promoter sequences can induce phenotypic change by altering HPI axis function (Kolber, Wieczorek and Muglia, 2008). Therefore, underlying genetic variation associated with these sequences, as well as *de-novo* mutations may have contributed to the results of intergenerational experiments.

Genetic variation in stress physiology may also affect how sensitive individuals are to stressful stimuli and their propensity to transmit phenotypic changes to their offspring. Anecdotally, variation in stress responsivity was observed when individual animals were exposed to alarm substance as part of the chronic stress regime (Chapter 3): while most individuals exhibited obvious responses characterised by erratic movements and prolonged freezing bouts, some individuals appeared to be relatively apathetic to the stimulus. In addition to genetic variation in HPI axis function and stress responsivity, the pathways underlying epigenetic inheritance itself may be subject to genetic influences (both paternal and maternal). Hypothetically, a given individual may be highly sensitive to stressful stimuli but may lack some genetically-encoded capacity to package environmental information in sperm, or if such information is successfully packaged, the maternal factors in the embryo may restrict the extent to which the information is implemented. When larval offspring (Chapter 3) were exposed to alarm substance, the thigmotactic response was not obvious in all control offspring sets, while neither was it ameliorated

in all paternal stress offspring sets. Genetic variation in either stress responsiveness or mechanisms of epigenetic inheritance may help to partly explain these discrepancies.

Another difficulty is that the experiments may have been confounded by the very mechanisms they were designed to glimpse. Chapter 3 showed that brief exposures to stress coincident with a critical reproductive stage are sufficient to induce detectable phenotypic alterations in the next generation. This suggests that experimental manipulations must compete with other sources of environmental variation in the parental generation for contribution to the offspring phenotype. For instance, animals housed in groups impose stress on one another through aggressive behaviour as part of dominance rituals (Fox *et al.*, 1997). As animals were not reared in isolation, exposure to such social stress long before an experiment may already have altered the epigenetic repertoire of the animal. There may even be epigenetic mechanisms that mask environmental effects by more direct counteraction. For instance, in the case of offspring born to stressed mothers in Chapter 2, it is possible that epigenetic mechanisms could work to counteract deleterious effects of early parturition, thus limiting the effects of maternal stress on phenotype in adulthood.

In the case of the paternal stress experiments, both genetic and environmental / epigenetic variation may have come from the maternal environment. Previous work has shown that maternal social stress influences cortisol levels and gene expression in subsequent offspring in zebrafish (Jeffrey and Gilmour, 2016a), while temperature stress has recently been shown to alter the transcriptional landscape of oocytes and behavioural phenotypes of offspring in cod (Colson *et al.*, 2018). Maternal variation was not examined in the paternal stress experiments, although males were assigned randomly to females.

Technical and methodological limitations

In addition to stochastic variation, it is important to acknowledge some of the technical and methodological limitations. The project relied heavily on behavioural tools for phenotypic characterisation of larval and adult offspring. Although this project has illustrated the great utility of behavioural phenotypes to study stress responses in larval and adult fish, numerous drawbacks and uncertainties remain in the measurement and interpretation of fish behaviour. For instance, while the

avoidance of light observed by the adult zebrafish used in this project corroborates with several previously reported observations, conflicting results are reported elsewhere in the literature, with data from many laboratories reporting the opposite trend (Maximino *et al.*, 2010). Research into larval behaviour is undoubtedly an area which warrants further development, as there is a need to expand the repertoire of simple, robust behavioural phenotypes available to stress researchers.

Furthermore, a broader array of non-behavioural stress phenotypes is required for more reliable characterisation of intergenerational effects. Measurement of larval heart rate, for which tools have recently been developed (De Luca *et al.*, 2015), could provide a promising additional stress response phenotype to complement behavioural and endocrine measures.

Lack of mechanistic insight

Perhaps the most obvious limitation of the work is the lack of mechanistic insights at the molecular and epigenetic levels. Dysregulation of several transcripts associated with HPA/HPI axis has been implicated in stress-induced pathological states, some of which were described in Chapter 1 (e.g. NR3C1, 11β-HSD). It therefore would have been pertinent to examine the effect of parental stress on transcriptional activity of genes associated with stress response physiology. Some preliminary attempt was made to examine transcriptional alterations in paternally-stressed zebrafish larvae by RT-qPCR, but these investigations were not completed. The gene selected for study was POMC, which encodes a prohormone for ACTH and is a key mediator of the HPA axis stress response (Wu *et al.*, 2014). In addition to its fundamental role in regulation of the stress response, it was considered pertinent to examine this gene because its dysregulation has been observed in the context of stress pathologies. For instance, transcription of POMC has been shown to be sensitive to methylation-induced silencing in response to early life stress in mice (Wu *et al.*, 2014), while similar epigenetic dysregulation of POMC has been observed in a rodent model of foetal alcohol syndrome and was found to be heritable via the male germ line (Bekdash, Zhang and Sarkar, 2014).

POMC expression was measured by TaqMan RT-qPCR in 5-DPF zebrafish larvae derived from fathers exposed to ‘OSS’ (onset spermatogenesis stress) as described in Chapter 3, and larvae from control

fathers. Although there was not a significant effect of paternal stress on larval POMC expression levels, there was a trend towards higher POMC expression in paternal stress offspring (Fig. 1). Higher POMC expression would suggest an overactive HPI axis, which, via a positive feedback mechanism, may result in blunted stress responses. The result must be interpreted with caution, however, because (1) the difference in relative gene expression level was not significant and (2) samples were pseudoreplicates, rather than true replicates (fathers from each group were housed together). Nevertheless, the observed trend suggests a larger investigation of transcriptional changes associated with paternal stress would be a worthwhile endeavour. Expression of other core HPI axis genes, such as NR3C1 (glucocorticoid receptor) and 11β-HSD2 may also plausibly be under dysregulation in paternally stressed offspring, and thus promising targets for a larger gene expression study.

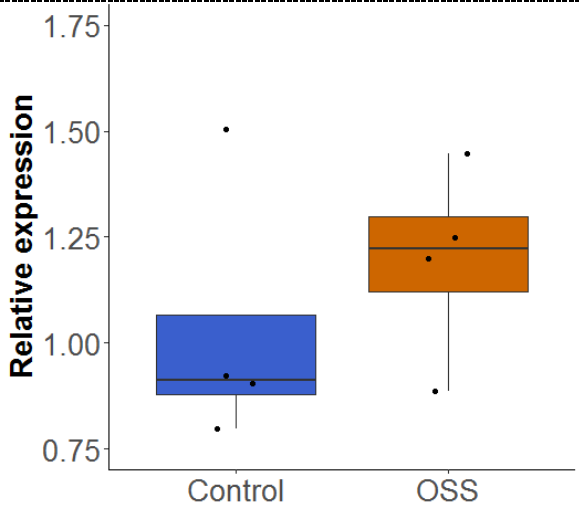

Fig. 1. Relative expression levels of proopiomelanocortin (POMC) in 5-days post-fertilisation offspring derived from control (blue) and onset-spermatogenesis stress (OSS) fathers. GAPDH was used as a housekeeping control, by which a relative POMC expression level (Δ-CT) was calculated for each sample. The final relative expression value, Δ-Δ-CT, constitutes Δ-CT of each sample relative to the mean Δ-CT of the control group.

Additionally, an attempt was made to characterise microRNA expression in sperm from stressed and non-stressed zebrafish using the Affymetrix miRNA array platform, but no data were obtained due to a technical failure and insufficient sample quantity to repeat the experiment. However, the miRNA array was attempted using samples which were not enriched for spermatozoal RNA, and thus even if the array

had succeeded in yielding data it is possible that the detection of stress-induced alterations to spermatozoal RNA could have been hampered by the inevitable abundance of transcripts from other cell types. Therefore, the decision was made to develop the spermatozoal enrichment protocol described in Chapter 4. The miRNA array was not repeated with enriched samples, however, as RNA yields subsequently obtained were substantially lower than those obtained from non-enriched samples and not sufficient for the input requirements of the array.

Although it does not compensate for a lack of mechanistic insight, I will pose extensive and detailed mechanistic hypotheses in the following section.

Future directions for the study of environmental epigenetic inheritance in teleost fish

Many of the above limitations can be addressed in the course of future experimental work. Of prime interest, however, are the possible mechanisms underpinning intergenerational effects at the molecular level. Given that paternal intergenerational effects in zebrafish comprised the most substantial body of work, I will focus exclusively on future work pertaining to this aspect of the project. As demonstrated in Chapter 4, it is now possible to obtain enriched zebrafish spermatozoal RNA in usable quantities for sequencing experiments. Together with phenotypic characterisation of paternal effects, this paves the way for further studies to test hypotheses about the possible role of paternal RNA in early embryonic development. Meanwhile, additional questions pertain to other factors that may influence epigenetic inheritance, for instance environmental factors other than stress, and the interplay between epigenetics and the underlying genetics.

Does sperm remember?

From a mechanistic perspective, the most stimulating questions surround the language in which intergenerational messages are relayed and implemented. Are paternal intergenerational effects mediated by the repertoire of RNA in sperm cells? If so, which transcripts do they interact with in the early embryo and how do these interactions affect the developmental trajectory of the organism? These

questions can be addressed in part using an experimental framework established in rodent models. Zebrafish, however, can move a step beyond rodents thanks to their external development which affords the opportunity to more easily observe early developmental processes and thus interrogate the mechanisms of intergenerational effects throughout the embryonic development of the organism.

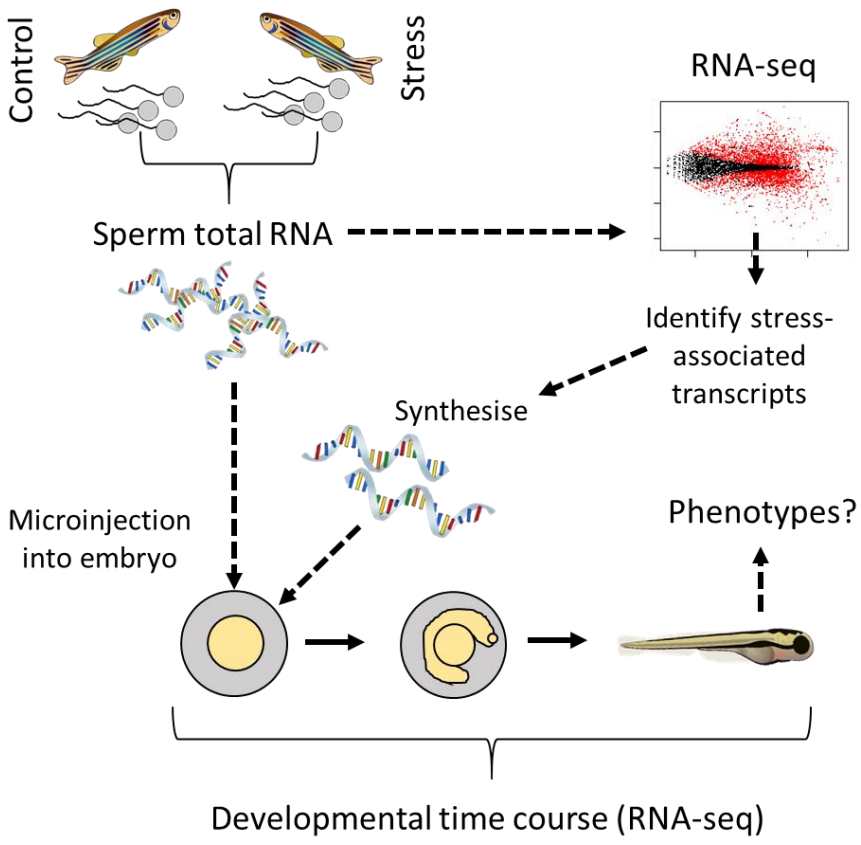

Figure 2. A proposed experimental framework, partially adapted from rodent models, to test hypotheses pertaining to the role of spermatozoal RNA in intergenerational epigenetic effects.

Assessing whether spermatozoal RNA is altered by stress could be achieved by RNA sequencing of sperm samples from stressed and non-stressed males, for which the groundwork has already been achieved (Chapter 4). The question could be more fully addressed with a functional genomics approach – by microinjection of total RNA or candidate RNA molecules (identified via RNA-seq) into otherwise normal early-stage embryos and examining the phenotypic consequences, as has been demonstrated in rodent models of paternal stress (Grandjean *et al.*, 2015; Rodgers *et al.*, 2015). If RNA is delivered to

embryos and is responsible for differences in phenotype, the next question is would pertain to how these changes are enacted.

Early embryos are transcriptionally quiescent, relying principally on maternally-derived RNA and proteins to direct developmental processes until the zygotic genome is activated around three hours after fertilisation (Despic *et al.*, 2017). Before this, however, during a period termed the maternal-zygotic transition (MZT), embryos undergo genomic reprogramming in which parental DNA methylation is shed and a fresh methylome established. At this stage, zebrafish embryos exhibit a remarkable molecular behaviour in that the pattern of DNA methylation established is almost identical to that of the sperm methylome (Jiang *et al.*, 2013; Potok *et al.*, 2013). It has been suggested that this process could possibly mediate epigenetic inheritance, and that it could be guided by paternally-inherited small RNA species (Jiang *et al.*, 2013). There is therefore a need to examine the transcriptional and DNA methylation profiles of embryonic offspring from paternal stress experiments, especially around the time of zygotic genome activation to examine the possible interactions between paternal stress, inherited RNA, and genomic reprogramming. A time-course experiment of embryo transcriptional profile from fertilisation until zygotic genome activation would be particularly insightful in delineating the possible influence of paternal RNA on the embryo's methylation status and developmental trajectory.

Does sperm forget?

Given the remarkable sensitivity of zebrafish to intergenerational alterations enacted in response to acute paternal stress (Chapter 3), it would be reasonable to hypothesise that the sperm epigenome is not permanently changed by stress and can revert to previous states if conditions become more favourable (an idea briefly discussed in Chapter 1). This could be tested by means of a time-course experiment, interrogating sperm molecular composition during a period of stress and at defined timepoints over several subsequent spermatogenic cycles after the stressful period has ended. A related, perhaps more obvious question is for how many generations does an intergenerational effect persist in zebrafish? Again, simple experimental approaches could be applied to address this and other questions.

Epitranscriptomics – a note on a new frontier in molecular embryology

While heritable noncoding RNA has already come to comprise a highly active area of research, more recently attention has turned to chemical modifications of RNA, similar to DNA methylation. The best-studied modification thus far is 6-methyladenosine, a form of methylation (Hsu, Shi and He, 2017).

RNA methylation has recently emerged as a regulatory mechanism of potential importance in early embryonic development, and its influence on the timing of MZT in zebrafish embryos has already been demonstrated (Zhao *et al.*, 2017). Overall however, the functions of RNA methylation in the early embryo are under-explored, and may include signalling for miRNA binding, degradation, translation, or alternative splicing (Roundtree *et al.*, 2017). Recent work which suggests that RNA methylation is environmentally-responsive (Castro-Vargas *et al.*, 2017) raises the question of whether the early embryo's transcriptional landscape can actively respond to the environment via methylation-dependent processes. For instance, environmentally-induced changes could arise in the form of differences in splice variants or degradation. Such changes may initiate a cascade which alters the early transcriptional landscape, leading to an altered methylome and transcriptome following zygotic genome activation (ZGA), and ultimately influencing phenotype.

It is also possible that RNA in spermatozoa could possess methylation profiles which are environmentally-responsive, or that sperm factors delivered to the embryo alter the epitranscriptional state of maternal transcripts, subsequently inducing cascades which alter developmental programming.

Potential role of extracellular vesicles in the epigenetic modification of sperm cells

So far, the discussion of potential mechanisms has largely focused on the possible effects of spermatozoal RNA or associated modifications on the dynamics of the early embryo. However, an equally interesting question pertains to the mechanism(s) by which germ cells could be altered by environmental exposures. Although evidence from rodent models (e.g. Gapp *et al.*, 2014) and indeed human studies (e.g. Marczylo *et al.*, 2012) have shown that environmental exposures alter spermatozoal RNA content, the specifics of how this material is integrated have not been established, although recent advances point to a prominent role of extracellular vesicles (EVs).

EVs are a heterogeneous group of cell-derived membranous structures with several established roles in intercellular communication (van Niel, D'Angelo and Raposo, 2018). They have been observed in physical association with spermatozoa in vivo and appear to be important for their maturation (Johnson *et al.*, 2015). For instance, they have been shown to be involved in the transfer of proteins from the epididymis to the sperm surface (Tosti *et al.*, 2001).

Recently, an essential role epididymis-derived small RNA was revealed for successful mammalian development. ICSI-produced zygotes using sperm derived from the proximal epididymis (where sperm begin epididymal passage) were inviable but could be rescued by injection of small RNA derived from the caudal epididymis (where sperm finish their passage), suggesting that functionally important RNA is delivered to spermatozoa during their passage through the epididymis (Conine *et al.*, 2018). The workers proceeded to show that incubating pre-caudal sperm with caudal-derived extracellular vesicles (EVs) altered the transcriptional composition of the sperm, specifically increasing the miRNA content, suggesting that RNA is delivered to sperm via EVs derived from the epididymis (Sharma *et al.*, 2018). Importantly, EVs would allow the composition of sperm cells to be modified even when the spermatozoon itself has completed its maturation, meaning that the spermatozoal composition could be altered, or may even be more susceptible to alteration, while in their mature form.

However, whether EV-derived RNA actually enters the spermatozoon by membrane fusion, or whether it remains within EVs which are tethered to the membrane surface (or if there is a combination of both) does not appear to be established. If carried within 'tethered' EVs, RNA would have the opportunity to enter the oocyte during fertilisation via fusion of EVs with the egg membrane. Incidentally, such EV-mediated delivery of RNA would not require the spermatozoon itself to succeed in fertilisation. If multiple sperm could deliver EVs to the oocyte in this way, this would have substantial implications for the father's overall contribution to the progeny and would thus have great relevance to evolutionary biology.

Exosome-mediated intercellular communication is conserved throughout evolution (van Niel, D'Angelo and Raposo, 2018) and the transfer of miRNA in exosomes has been shown to play regulatory functions in the zebrafish nervous system (Xu *et al.*, 2017). Teleost fish do not possess an epididymis,

but sperm are stored and nourished within the efferent ducts (Boj, Chauvigné and Cerdà, 2015) where transfer of EVs similar to that observed in a rodent model (Sharma *et al.*, 2018) could theoretically take place. To my knowledge, however, there has been no investigation carried out into the possibility that EVs could mediate the stable modification of piscine germ cells, or whether environmental inheritance in fish is mediated by a different set of mechanisms. Indeed, as zebrafish embryos have been demonstrated to inherit paternal DNA methylation profiles (Jiang *et al.*, 2013; Potok *et al.*, 2013) and altered sperm DNA methylation has been implicated in intergenerational effects of bisphenol-A in zebrafish (Lombó *et al.*, 2015), there would appear to be a substantial contribution of sperm DNA methylation to the language of epigenetic inheritance in fish. Because epigenetic reprogramming occurs before spermatogenesis is complete, such modifications would not be likely to occur in mature sperm cells. However, instructions which direct alterations to sperm methylation could be delivered to developing sperm cells via EVs secreted by the Sertoli cells which enclose the germ cells throughout their development.

Investigation of other environmental and genetic influences

Thus far, at least two other forms of environmental variation: sperm competition (Zajitschek *et al.*, 2014) and toxicant exposure (Lombó *et al.*, 2015) have been demonstrated to induce paternal intergenerational effects in fish. Other manipulations which may induce interesting effects may include abiotic factors such as temperature and salinity, pathogen exposure, environmental complexity, different diets, or social stress. Some of these aforementioned manipulations have already been investigated for their potential to induce paternal effects in rodent models, e.g. diet (Grandjean *et al.*, 2015). In zebrafish, the effects of social stress could be studied by exploiting their natural dominance hierarchies, however, such an approach may be confounded in that individuals which become subordinate may be genetically pre-disposed to this fate.

In addition to behavioural neuroscience and developmental biology, zebrafish are steadily emerging as a model in infection biology and immunity – another area in which epigenetic inheritance could be highly relevant. Stress and immunity are also intrinsically linked, as lowering of immune defences is an important consequence of heightened stress (Lupien *et al.*, 2007). It could be hypothesised therefore

that elevated stress also induces changes to immune function which themselves have intergenerational ramifications, perhaps altering the immune function of the subsequent offspring. Some attempt was made to measure an immune response in zebrafish larvae following immersion in Lipopolysaccharide (LPS), a pathogen-associated molecular pattern (PAMP) secreted by gram-negative bacteria and a potent immune system stimulant in mammalian models (both in vivo and in vitro) (Steven *et al.*, 2017). LPS-induced immune response was to be measured by fluorescence produced by a tag for reactive oxygen species (ROS – generated in the immune response, Fig. 3), but consistent measurements were not obtained. Nevertheless, if methods of immune response measurement could be optimised, immune function – a critical component of vertebrate physiology – would be a promising avenue to explore in future intergenerational studies.

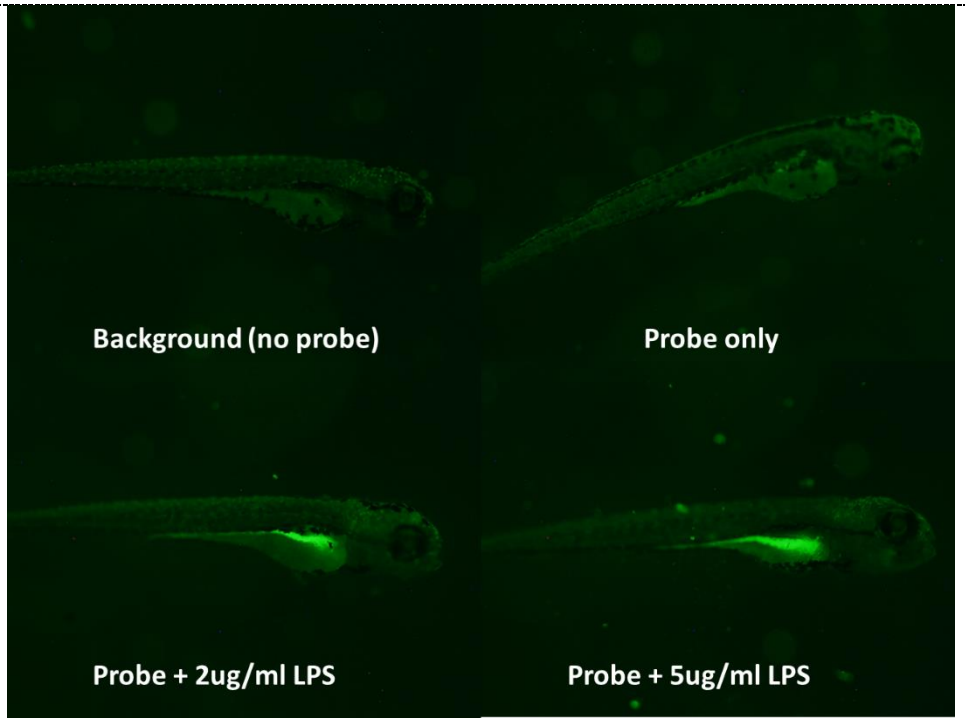

Figure 3. Zebrafish larvae treated with different concentrations of bacterial lipopolysaccharide (LPS) and a probe (H_2-DCFDA) which converts to fluorescent DCF upon exposure to reactive oxygen species (ROS), thus betraying the occurrence of an oxidative burst in response to LPS.

Aside from investigations into other environmental influences, experiments could be designed to examine the extent to which epigenetic inheritance has a genetic basis. Such an experiment would rely

on pre-existing variation in quantifiable behavioural traits (termed personality traits in this context), and could assess, for example, whether an individual's position on a boldness-shyness continuum (Ariyomo, Carter and Watt, 2013) affects its sensitivity to a stressor and the likelihood of elevated stress leaving phenotypic traces in its subsequent offspring. Genome-wide analyses would serve as invaluable resources and could be applied to examine single-nucleotide polymorphisms or copy number variants which could be associated with sensitivity to stress or propensity for epigenetic inheritance.

Concluding remarks

The work carried out in this project has shown that fish are susceptible to intergenerational influences on stress response in a similar manner to humans and other mammals, and most significantly has shown for the first time that paternal stress impairs offspring stress responses in a teleost fish. The project has been hampered by numerous limitations, comprising both stochastic variation and technical limitations, although such limitations could be overcome in future studies and even form the basis of new studies. In addition to laying the technical foundations for future studies of RNA-mediated epigenetic inheritance in fish, I have posed several new questions and hypotheses and suggested numerous areas in which the field of research could be developed – both inwards towards a deeper mechanistic level of molecular embryology, and outwards to encompass other fields such as immunity and population genomics. Ultimately, this thesis demonstrates that small teleosts can be of great utility in the study of long-term and intergenerational effects of stress. It is hoped that future work can be carried out to open the lid on gametes of embryos in search of the mechanisms underlying these enigmatic phenomena.

Appendix 1

Ionic stress prompts premature hatching of zebrafish (*Danio rerio*) embryos

Abstract

Ionic homeostasis is an essential component of functioning cells, and ionic stress imposed by excessive salinity can disrupt cellular and physiological processes. Therefore, increasing salinity of aquatic environments – a consequence of global climate change – has the potential to adversely affect the health of aquatic animals and their ecosystems. The ability to respond adaptively to adverse environmental changes is essential for the survival of species, but animals in early embryonic stages may be particularly vulnerable as they cannot easily escape from such conditions. Herein, the effects of increasing salinity on the mortality and hatching time of zebrafish (*Danio rerio*) embryos were investigated. Increasing salinity significantly affected mortality after 24 hours of exposure beginning from < 2 hours post-fertilisation, with 10 parts per thousand (ppt) inducing complete lethality. The 24-hour LC50 of NaCl to embryos was estimated to be approximately 5.6 ppt. Interestingly, 5 ppt, a test concentration only slightly lower than the LC50 induced earlier hatching than at lower concentrations. This earlier hatching was also observed even when exposure was commenced at later stages of embryogenesis, despite later-stage embryos not suffering appreciable mortality in response to salinity. The results suggest that earlier hatching is plastic response which may function to enable embryos to evade unfavourable conditions.

Introduction

Salinity is an important feature of aquatic environments, and maintaining ionic homeostasis is crucial for optimal function of cellular and physiological processes (Hwang and Chou, 2013). Ionic stress, induced by increases in salinity, therefore has the potential to disrupt these processes. Increasing salinity of freshwater environments is exacerbated by rising sea levels as a consequence of global climate change (Vineis, Chan and Khan, 2011) and by road salt run-off (Kaushal *et al.*, 2005), and poses an important threat to aquatic ecosystems. For instance, a long-term monitoring study found that salt concentrations of freshwater streams in Milwaukee, USA, frequently exceed 1000 mg/L (1 ppt) and extended to nearly 7000 mg/L (7 ppt) during road salt application periods in February-March – a range which included concentrations that were lethal to adult fathead minnows in a chronic bioassay (Corsi *et al.*, 2010). Freshwater habitats face an additional source of increased salinity in the form of saltwater intrusion as a consequence of intensive aquaculture, which is especially prominent in southeast Asia as a consequence of intensive shrimp farming (Páez-Osuna *et al.*, 2003). Therefore, it is important to characterise the potential effects of increasing environmental salinity on aquatic organisms.

Fully-developed animals are able to respond adaptively to unfavourable chemical environments via physiological or behavioural responses (e.g. escaping from the adverse environment), however animals at earlier stages of development – particularly embryos – may be far more sensitive to adverse environments. Osmoregulation in teleost fishes is carried out by the coordination of several organs and organ systems (e.g. gills, kidneys, urinary system), but these structures are not differentiated in early embryos which instead rely on ion pumping activity by ionocytes covering the skin (Pittman *et al.*, 2013). Importantly, developing embryos are immobile while confined to the egg casing and thus cannot easily escape from unfavourable conditions as adult animals can.

The zebrafish (*Danio rerio*), a freshwater fish, is an ideal model system for the effects of physiological stress on the early embryo given that eggs are fertilised externally and can be easily observed throughout early development. Their rapid development into free-swimming larvae within the first five days of life also makes them suitable for high-throughput experiments. Zebrafish are thought to be tolerant to a

range of salinities in their natural environments which have been recorded to range from 0.1-0.6 ppt, thus technically extending to brackish conditions (> 0.5 ppt) (Lawrence, 2007). However, this tolerance range has not been well-characterised. Increasing rearing salinity has previously been shown to induce higher mortality on the embryos of non-marine fish including zebrafish (Sawant, Zhang and Li, 2001; Haque *et al.*, 2014) and medaka (Rosemore and Welsh, 2012), but the propensity of embryos for plastic responses under ionic stress conditions has not been investigated. One such trait which may be subject to plasticity is hatching – an important life history event in egg-laying species and exhibits plasticity in response to biotic and abiotic stress (Warkentin, 2011). In zebrafish, hatching time is mostly determined by the rate of development (largely controlled by temperature (Schirone and Gross, 1968)), but can also be influenced by light regimes, suggesting a role of circadian chemistry in the regulation of hatching (Villamizar *et al.*, 2012). Overall, however, hatching plasticity has not been extensively studied in zebrafish and has not been evaluated in the context of salinity.

This study first aimed to (1) characterise the effect of salinity on mortality of zebrafish embryos, and (2) assess the effects of different salt concentrations on the timing of hatching.

Results and Discussion

Initially, 24-hour mortality of embryos was assessed using four NaCl concentrations (0.2, 2, 5, and 10 parts per thousand), the lowest of which was the concentration of aquarium water. Exposure commenced < 2 hours postfertilisation (hpf), when embryos were in the 4-64-cell stage. The relationship between concentration and mortality was modelled using a generalised linear model, for which the goodness of fit was assessed using pseudo R^2, calculated as the inverse of the ratio of the residual deviance to the null deviance. The model had a pseudo R^2 of 0.87 and showed a significant effect of concentration on 24-hour mortality ($p < 0.001$). The 10 ppt concentration was lethal to all embryos, 5 ppt induced moderate mortality (up to 50%), while mortality among 2 ppt embryos was noticeable but did not exceed 20% (Fig. 1). As predicted from the model, the LC50 of NaCl was approximately 5.6 parts per thousand.

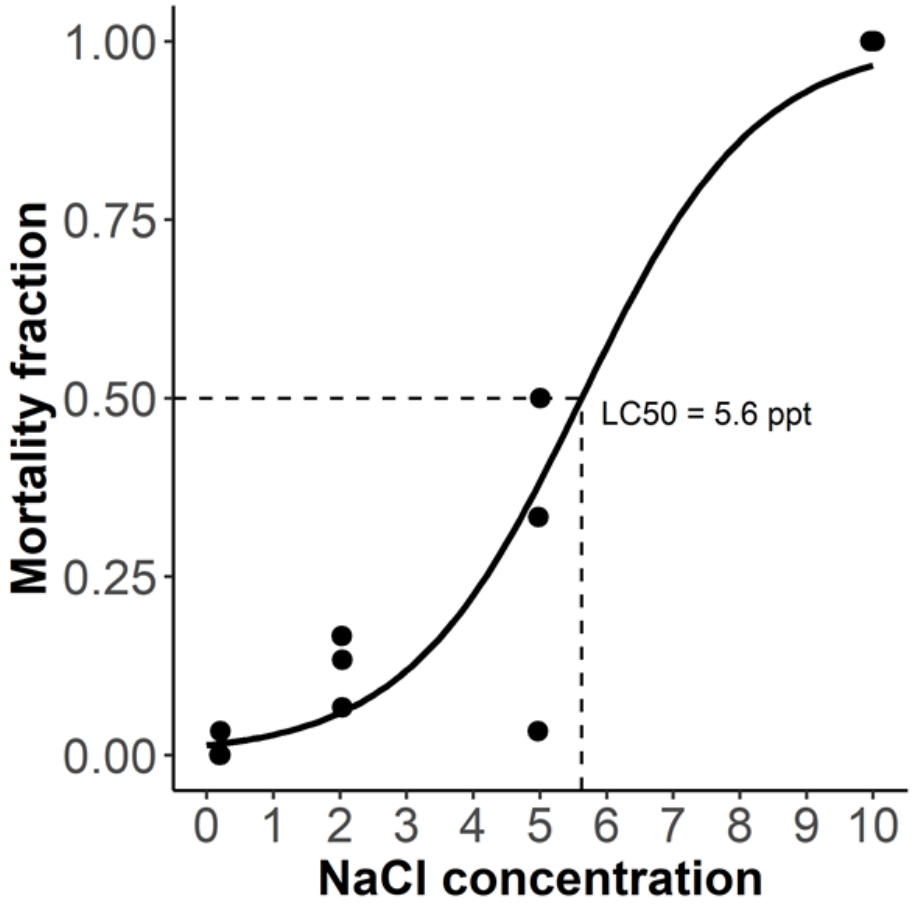

Figure 1. Concentration-response relationship between salinity (salt concentration in parts per thousand) and embryo mortality % after 24 hours of exposure beginning from < 2 hours post-fertilisation. N = 3 dishes of 30 embryos per treatment level, with each point representing a single dish. Curve fit and predicted LC50 were obtained using a generalised linear model.

The results are generally concordant with previously published experiments, e.g. Haque et al. (2014) showed that 21% of embryos survived to hatching in 6 ppt salinity while none survived in 10 ppt. One brood exposed to 5 ppt which did not exhibit appreciable mortality at 24 hours, which may be reflective of genetic differences in osmoregulatory capacity of early embryos. However, as within-brood replication was not performed, the reason for this discrepancy is unclear.

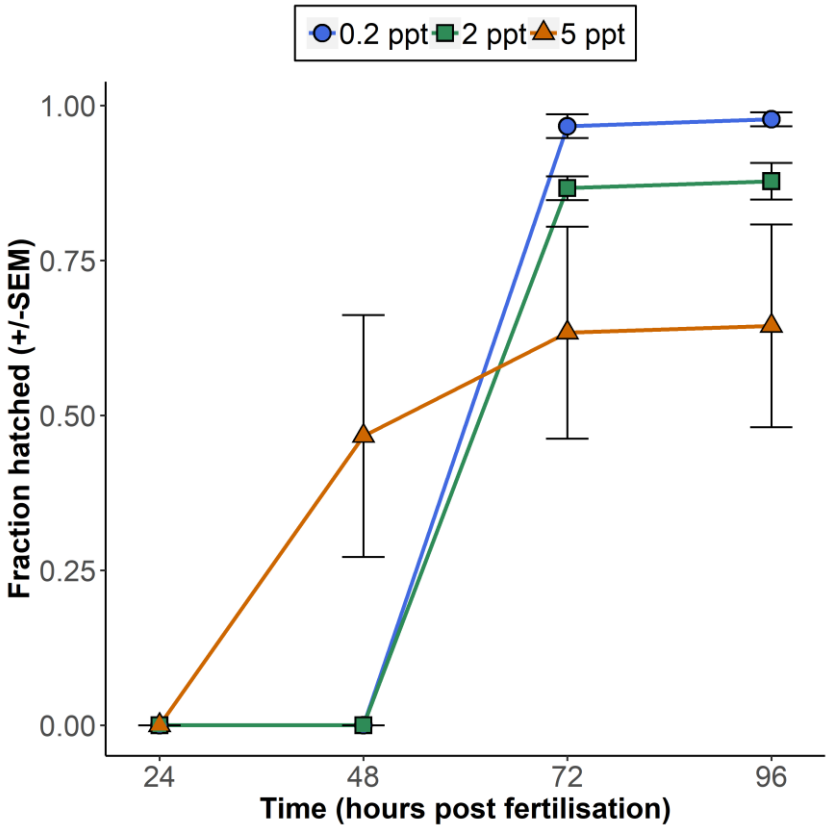

Figure 2. Proportion of zebrafish embryos hatched between 24- and 96-hours post-fertilisation while exposed to three salt concentrations up to 5 parts per thousand (ppt) in petri dishes at 25°C, with exposure beginning from < 2 hpf. N = 3 dishes of 30 embryos per treatment level, with each point representing the mean of three dishes.

Although 5 ppt resulted in fewer embryos hatched at 72 hpf, embryos reared at this concentration hatched earlier than their lower salinity counterparts: hatching at 0.2 and 2 ppt was not observed until 72 hpf, while most of the surviving embryos exposed to 5 ppt hatched 24 hours earlier than at these lower concentrations, at 48 hpf (Fig. 2). For statistical evaluation of the interaction between concentration and hatching time, a linear mixed model was fitted which accounted for repeated measures from the same dishes. This model showed a significant interaction between 5 ppt salinity and time (T-test with Kenward Roger approximation of degrees of freedom, $T^{24} = 2.1$, $p = 0.045$).

A previously study demonstrated that the lethality of salinity to zebrafish embryos depended on the developmental stage at which they are exposed (Sawant, Zhang and Li, 2001). Embryos become more tolerant of salinity from the blastula and gastrula stages compared to the cleavage stage, presumably due to more advanced osmoregulatory capacities. Therefore, the present study also examined hatching

data from two additional experiments in which groups of embryos were exposed to 0.2 or 5 ppt salinity beginning from the late blastula stage or later (4- or 24 hpf depending on the experiment), and hatching was monitored between 48 and 72 hours. Concordant with the first experiment (Fig. 2), 5 ppt embryos showed increased hatching from 48 hpf (Fig. 3). A linear mixed model with time as a quadratic term revealed that hatching % was significantly higher at 5 ppt throughout the observation period (F-test with Kenward Roger approximation of degrees of freedom, $F^{1,13.3} = 18.4$, $p < 0.001$) and that there was a significant interaction between treatment and time ($F^{2,37.1} = 7.6$, $p = 0.002$). Five ppt salinity did not induce appreciable mortality as overall hatching success was not impeded (close to 100% for most 5 ppt dishes at 72 hpf). This suggests later stage embryos are resilient to a concentration that is lethal to earlier stage embryos and thus supports previously published observations (Sawant, Zhang and Li, 2001). Hatching at 0.2 ppt was noticeably lower at 72 hpf compared to the first experiment (Fig. 2), which may be attributable to other environmental differences relating to the embryos having been transferred from egg collection dishes to petri dishes at a later time (see *Materials and Methods*). Importantly, these data indicate that increased salinity still prompts earlier hatching even at later stages when embryos are supposedly more physiologically tolerant.

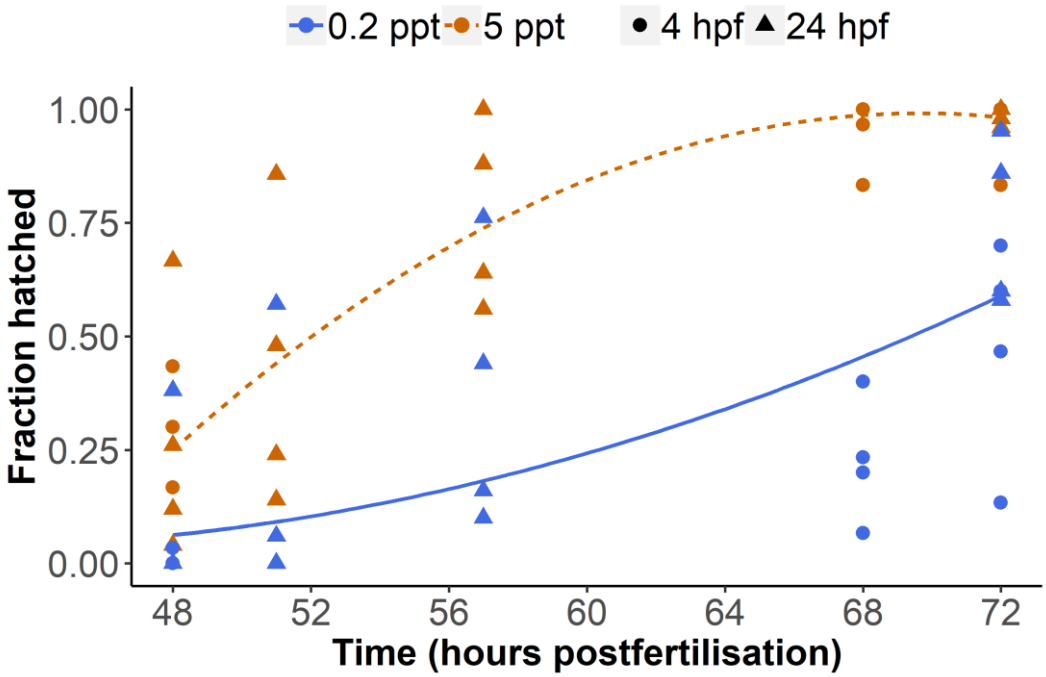

Figure 3. Proportion of zebrafish embryos hatched between 48- and 72-hours post-fertilisation while exposed to salt concentrations of 0.2 (blue) or 5 ppt (orange) in petri dishes at 25°C, beginning from either 4 hpf (circles) or 24 hpf (triangles) depending on experiment. N = 8 dishes of 30 embryos per salt concentration, with each point representing a single dish. Curve fits derived from a linear mixed effects model (time as quadratic term).

Overall, the results of the present study demonstrate that zebrafish embryo hatching is plastic in response to potentially stressful or lethal changes in salinity. Premature hatching has previously been observed in tree frog embryos upon snake attack (Warkentin, 1995, 2011) and in zebrafish embryos in response to environmental toxicants (Liang *et al.*, 2017), suggesting that earlier hatching is an adaptive response which allows the animal an opportunity to escape unfavourable conditions. As the salt concentration which induced earlier hatching induced mortality of early embryos, the results of this study are in alignment with this paradigm. It could be inferred that as external ion concentrations threaten to exceed the capacity of ionocytes to regulate internal osmolarity, 'emergency' pathways are triggered which accelerate the hatching process. Hatching is triggered following induction of hatching enzyme synthesis (Yamagami, 2009), and thus such emergency response pathways would likely involve mechanisms by which hatching enzyme synthesis is induced earlier or at greater levels than normal.

Given their higher tolerance to salinity, this response would be less urgent for later stage embryos, however it would still provide an advantage assuming heightened salinity negatively affects other aspects of fitness.

It must be conceded that this study did not ascertain whether earlier hatching in response to increased salinity was driven by accelerated development rate (as is the case for temperature (Schirone and Gross, 1968)) or whether embryos hatched at an earlier developmental stage. As zebrafish hatching time appears to be controlled partially by light, via the circadian clock (Villamizar *et al.*, 2012), it is also possible that ionic stress induces earlier hatching by disruption of circadian clock chemistry.

It must also be conceded that the present study has examined the effect of salinity in only one strain of zebrafish (London wildtype). Under common husbandry practices, laboratory zebrafish are reared in low-salinity conditions (0.25-0.75 ppt) (Lawrence, 2007), and it is not known whether domestication has affected salinity tolerance relative to wild varieties, which may be tolerant of a wider range of salinities. From comparative studies of different laboratory strains and wild varieties, insight could therefore be gained into the effect of domestication on salinity tolerance. Furthermore, as zebrafish are thought to be tolerant of a range of salinities in the wild (Lawrence, 2007), the embryo responses observed in this study may not be entirely representative of stenohaline species, and it would therefore be pertinent to compare salinity-induced hatching plasticity across a range of stenohaline and euryhaline species.

In conclusion, the results of this study both support and expand upon previously published findings on the effects of salinity on early zebrafish embryos, showing that marked plasticity occurs in hatch timing in response to a concentration that is close to the LC50 for early embryos. The phenomenon warrants more detailed characterisation, as salinity-induced hatching plasticity could serve as a useful phenotypic reference for studies of osmoregulatory and stress response capacities of the early embryo. Furthermore, the results suggest that embryonic adaptive plasticity may be an important component of the response of aquatic organisms to changes in environmental salinity.

Materials and Methods

Adult London wildtype zebrafish were maintained in a UK Home Office approved facility in the Department of Animal and Plant Sciences, University of Sheffield. Collection of embryos from adult zebrafish was carried out under the UK Home Office project licence number 40/3704. As no NaCl exposures were carried out on animals older than 5 days post-fertilisation and no exposed embryos were kept beyond 5dpf, the exposure of embryos in the experiment did not comprise a regulated procedure as specified under the Animals (Scientific Procedures) Act (1986) and thus did not require specific approval.

Embryos were derived from adult London wildtype *Danio rerio* which were reared and housed on a recirculatory system with water heated to approx. 26°C. Animals were fed twice daily with live *Artemia* nauplii or commercial flake food. Egg collection dishes filled with marbles were placed in mixed sex tanks at approx. 1600 and were collected the following morning. Three clutches of fertilised eggs were derived from three separate sets of mixed-sex adult zebrafish, and each clutch was considered to be a biological replicate. Although the exact time of fertilisation could not be determined, zebrafish typically spawn at first light (Spence *et al.*, 2007), which was between 08.00 and 08.30 in aquarium where the present study was carried out. At less than 2 hours post-fertilisation (before 10.00), four sets of 30 embryos at the 4-64-cell stage (identified under light microscope) were taken from each clutch and were placed in lidded 90 mm petri dishes containing each of four salt concentrations (0.2, 2, 5, and 10 parts per thousand). The lowest was 0.2 ppt as this was the salinity of aquarium water with no additional salt. Higher concentrations were prepared by dissolving 'Instant Ocean' salt in aquarium water and concentrations were checked using a digital salinity meter. Embryo dishes were kept in a temperature-controlled room at 25°C on a 12:12 light / dark cycle, and were checked for mortality and hatching after 24, 48 and 72 hours of exposure.

For the experiments in which embryos were exposed to increased salinity at later developmental stages, embryos were collected in the same manner as described above, again with each set of embryos deriving from a separate set of adult zebrafish. Marbles were removed from the egg collection dishes and water

changed, and embryos remained in these dishes until being transferred to petri dishes for exposure to either 0.2 or 5 ppt NaCl. For one experiment, embryos were transferred at approx. 4 hpf (around 12.00 on day of egg collection), while for another experiment they were transferred at 24 hpf. The mixed model fit to this data did not reveal a significant effect of experiment on the response variable ($F^{1,13.9}$ = 2.6, p = 0.13).

Graphical generation and statistical analyses were carried out in 'R' version 3.5.1 (R Development Core Team, 2011). The generalised linear model for 24-hour mortality was fitted using the glm() function from the 'stats' package and LC50 value predicted form the model using the predict() function from the same package. Mixed effects models fit using the lmer() function from the 'lme4' package (Bates *et al.*, 2015) were used to model the interaction between concentration and time on hatching, with 'Dish ID' entered as a random factor. For analysis of hatching data of later stage embryos, time was included as a second-degree polynomial (quadratic) term. Model results were derived using the summary() and anova() functions with significance values (T-tests and F-tests with Kenward Roger approximation of degrees of freedom) obtained using the 'lmerTest' (Kuznetsova, Brockhoff and Christensen, 2017) and 'pbkrtest' (Hakekoh and Hojsgaard, 2014) packages. Curve fits shown in Fig. 2 were derived using the 'effects' package (Fox and Weisberg, 2018) and a linear mixed model.

Appendix 2

Examiner's joint Summary Report on the Thesis and list of Required Amendments

Prepared by Julia Schroeder and Vincent Cunliffe

Summary Report

The thesis describes a research project to investigate the phenotypic consequences of parental stress on offspring phenotypes for two teleost fish with distinct modes of reproduction: guppies and zebrafish. Female guppies are live-bearing and their eggs are fertilized in utero, enabling the impacts of gestational predation stress (GPS) experienced by mothers and offspring to be investigated. By contrast, zebrafish eggs are fertilized externally by sperm released by the male into the water, and they subsequently develop independently of their parents. The zebrafish studies focused on investigating the impacts of chronic and acute paternal predation stress on F1 offspring behaviour and endocrine function. The thesis also describes initial results to elucidate the molecular mechanisms underlying the phenotypic changes they observed. The introduction reviewed current understanding of biological responses to stress and highlighted epigenetic mechanisms, including RNA-based processes, that may be involved.

Preparations of Alarm Substance (AS) were used to elicit predation stress responses in guppies and zebrafish. The results show that exposure of pregnant guppies to AS reduced gestation time, reduced thigmotaxis of F0 mothers, and decreased locomotor activity of mature F1 progeny. Both chronic and acute exposure of adult male zebrafish to AS before mating with naïve females, led to their larval offspring exhibiting attenuated behavioural responses to AS. Chronic paternal AS treatment further reduced the level of cortisol induced by exposure of progeny larvae to AS or to physical stress. Acute exposure of fathers to AS attenuated thigmotactic behaviour of their progeny in juvenile stages. The candidate hypothesises that the intergenerational transmission of the phenotypes elicited by paternal

exposure to AS is mediated by RNAs, as is the case in rodents. Accordingly, the candidate developed a technique for purification of mature sperm from whole adult zebrafish testes in sufficient quantities to enable RNA samples to be prepared that can be sequenced. This technique is described in detail in the thesis. The thesis concludes with a General Discussion that sets the results in some context and identifies future directions in which this research could be taken. Overall the contents of the thesis are original and important contributions to understanding biological responses to stress and discovering how stress-dependent phenotypes are transmitted to progeny by mechanisms that include functional modification of germ cells.

Required Amendments

The thesis would benefit from substantial re-structuring with particular focus on incorporating the Addenda and Appendices into the five main chapters. The thesis is structured into a series of Chapters: the General Introduction, the three Results Chapters and the General Discussion. The Results chapters are each written in the style of a stand-alone paper, with a tailored Abstract, Introduction, Materials and Methods, Results, Discussion and Concluding Remarks. This structure is consistent with the recommendations for the Alternative style of thesis and for publication as a series of papers, but each of these Chapters is accompanied by Addenda, and the General Discussion is accompanied by a series of Appendices. The contents of these addenda and appendices could be beneficially, logically integrated into the main Chapters. There are also several typos that can be corrected.

Chapter 1: General Introduction

This chapter provides a brief introduction to periconceptional stress and includes short sections on the biology of endocrine stress, its dysregulation in psychiatric conditions, and how animal models, including fish, are amenable to studies to identify some of the biological mechanisms mediating stress responses. A range of studies indicate that stress-induced phenotypes can be transmitted across multiple generations, and this chapter reviews some of the literature on intergenerational transmission and transgenerational inheritance of these phenotypes and some of the mechanisms that have been

elucidated. The sensitivity of the gamete-forming processes and gestation to external stressors is highlighted. However, in order to provide satisfactory context for understanding, and linking the three result chapters, these topics should really be reviewed in much greater detail, with clear indications and justifications for each results chapter. In particular, we suggest that

- The physiological mechanisms mediating stressor detection and mobilisation of endocrine and metabolic responses should be reviewed in more detail, including comparison of the timing and targets of adrenergic and HPA axis responses.
- The roles of known epigenetic mechanisms (e.g. DNA methylation, histone modifications, ncRNAs, the epitranscriptome, and the enzymes responsible for adding and removing covalent modifications to chromatin), in both development and in response to external environmental signals, should also be described in greater depth.
- The literature on the Developmental Origins of Health and Disease (DOHaD) should be clearly explained, and then reviewed in greater depth and its relevance to biological stressors, epigenetic mechanisms, the difference between acute and chronic stress in terms of symptoms, physiology, adaptations and consequences, and the emergence of chronic disease across the life course should be more clearly explained.
- Given that this project focuses on transgenerational effects, the biology of male and female germ cell development and differentiation should be properly introduced in this chapter. The reproductive biology of zebrafish and guppies should also be compared, contrasted, and related to any relevant similarities and differences in their behavioural ecology / life histories, e.g. interactions with predators and availability of food, as appropriate.

Including diagrams or figures in the Introduction that illustrating interactions between components of relevant physiological or developmental processes (e.g. stress responses, epigenetics, gametogenesis) would be very helpful to enable the reader to understand the complexities involved. These three aspects should help to develop the introduction to set the scene for the three results chapters.

Chapters 2, 3 and 4 – General Points

- Figure Legends are very brief and many of the Figures are not correctly labelled or formatted. Please reformat figures in line with guidelines for as publication (no main titles, give clear labels (a,b,c) for different panels, etc).
- Explicit description of sample sizes for the different experiments, and experimental treatment groups are needed as they differ from the numbers that undergo treatment.
- Descriptive statistics, including the means, variance are missing and need including.
- The descriptions of the statistical models used are vague, as they aim to describe multiple models in one go. However, because the response variables are on different levels of nestedness (some are summary statistics and others are individuals), it is essential to be explicit about the properties of the models used.
- The reported statistical results (comparisons between models including a fixed effect and those without) are not adequate nor sufficient to test the hypotheses.
- The complete model results including effect sizes (parameter estimates for both, fixed and random effects) are not reported. These need to be presented, probably best in tables as is customary.
- Language describing the results needs to be revised with respect to whether an effect is statistically significant (=existing) or not (=then there is no effect). In the latter case, it should not be written as if there were an effect – however, you can write about effect sizes if you wish so. This is difficult because the testing of the hypothesis requires the interaction term to be significant. This should be better explained in methods and results in both Chapter 2 and 3.

Chapter 2 Specific Points

1. Page 21: after 23 days of exposing pregnant females to AS, then both mothers and progeny have been exposed. Modify the text to acknowledge this.

2. Page 22: Figure 2 is a validation of AS activity and should be included in the section on page 20 in the section on “Alarm Substance Extraction and Validation”.

3. Page 24: Statistical analyses of offspring parameters used “linear mixed models…..” but models need to be described and tables of data should be presented – for all data in the thesis where this approach was taken. For example, in Figure 3 (p.25) an accompanying table showing parameter estimates with standard errors should be included.

4. Page 27: Data in Figure 5: the contribution of Kelle Holmes should be acknowledged.

5. Chapter 2 Addendum investigates the impact of high versus low diet on growth rate. This could be included within Chapter 2 because Figure 3 shows a significant reduction in offspring growth rate up to 10 days post-partum. One possibility is that GPS offspring consume less food. The your experiments in the Addendum refute this hypothesis and could thus be included after Figure 3 within the main chapter.

Chapter 3 Specific Points

1. Chapter 3 Addendum 1. Whilst these experiments might not fit well within the narrative flow of the other experiments in Chapter 3, they are still nevertheless experiments to investigate the impacts of chronic and acute stress on paternal and offspring behaviour and offspring life history traits, and as such should be included within the main chapter. Justify the experiments!

2. Chapter 3 Addendum 2: the *pomc* expression study needs more explanation of why it was done and how the results might be interpreted. It would fit better within the General Discussion as an initial experiment as part of a discussion about the direction of future research.

Chapter 4 Specific Points

This chapter describes results that attempt to take forward some of the conclusions of Chapter 3 and is predicated on the hypothesis that sperm RNAs are mediating some of the phenotypic changes observed in offspring of chronically and acutely stressed fathers, as has been observed in rodents. The Introduction should be used to frame this Chapter as an attempt to test this specific hypothesis, and the key published papers in rats and mice that provide evidence broadly supporting this hypothesis can be cited here too. The introduction should also refer back to the description of the spermatogenic cycle in fish and mammals that were described in the General Introduction.

Chapter 5 Specific Points

1. The General Discussion is a good attempt to place the key findings in this thesis in an appropriate scientific context. The findings are related to those of key papers cited in the General Introduction and Results chapters, and broadly speaking this chapter helps the reader to make sense of some of the data. But discussion of DOHaD, for example, is too brief and needs more extensive discussion, especially given that the candidate has documented examples of both maternal and paternal intergenerational transmission of stress-induced phenotypes. Pre-existing epigenetic and genetic variation, and limitations of experimental design and methodology are considered, as potential sources of phenotypic variation. A more detailed discussion of potential mechanisms that may be responsible for intergenerational or transgenerational inheritance of phenotypes, should be developed in this final discussion, considering the range of different molecular mechanisms that have been demonstrated to exhibit stress-responsive changes in the epigenome, methylome, or transcriptome, should be included. Recent studies implicating the epididymis as a source of sperm miRNAs in the mammalian testis, might be considered for further discussion (e.g. https://www.ncbi.nlm.nih.gov/pubmed/30057276 or https://www.ncbi.nlm.nih.gov/pubmed/30057273).

2. The contents of the General Discussion Appendix 1 (on Epigenetics) and Appendix 2 (Non-epigenetics-based mechanisms) should be moved to the General Introduction. Appendix 3 reveals that increased salinity promotes premature hatching. Could these data be moved to a Results chapter?

Bibliography

References should be given in full within the Bibliography.

Additional

Page 2, *Addresses* should not be in capitals

Page 9: “or psychologically by a perceived external threat” - could also be a real threat

Page 10 footnote not a sentence (a the then)

Page 12 “Karl von Frisch” – no reference provided

Chapter 2+3+Addenda: Figures should not have titles. Provide figures in numbered order. Give effect sizes. Give sample sizes. Give full statistical results including fixed and random effects (including residual variances and a measure of precision).

Chapter 3: it is not always clear whether you speak of early or later live stages – make this explicit in results and discussion.

Appendix 3

List of amendments implemented in the corrected version of the thesis

Chapter 1

Requested changes

- Additional material from the published review on periconception stress (Ord et al. 2017), which was not originally included in the thesis, has been fully implemented into the introduction.
- Material from the two 'mini reviews' on definitions of epigenetics and non-epigenetic mechanisms of parental effects (originally included as appendices) have been integrated into the introduction.
- Physiological stress response mechanisms have been reviewed in more detail, including comparison of adrenergic and HPA axis stress responses.
- Specific epigenetic mechanisms have been described and reviewed in greater depth.
- Literature on Developmental Origins of Health and Disease (DOHaD) has been reviewed in greater depth, and the relevance of the concept to stress and epigenetic mechanisms has been explained.
- The biology of male and female gametogenesis has been reviewed and notable comparisons drawn between mammals and fish. Some attention has also been drawn to embryognesis.
- The reproductive biology of zebrafish and guppies has been compared and contrasted in relation to differences in their ecology.

- Additional diagrams and figures have been included to aid the reader in their understanding of relevant concepts.

Chapter 2

Requested changes

- Content formerly contained in ‘Addendum 1’ has been fully integrated into the main chapter.
- Figures updated – removed titles above plots. Also added sample sizes to plots where necessary.
- Model results tables are presented for major mixed effects models, including parameter estimates of fixed effect terms and variance estimates of random effect terms. Although results tables are not included for the smaller models (offspring weight and behavioural traits), details of all the models (fixed and random terms, sample size) are included in Table 3.
- It has been made clearer that the former Fig. 2 (now a component of the new Fig. 1), showing the effect of alarm substance on naïve animals, comprises methods validation.
- Text in methods describing GPS experiment has been modified to acknowledge that both mothers and embryos were exposed to alarm substance.
- The contribution of Kelle Holmes has been acknowledged in Fig. 5.
- Inferences made from non-significant results have been toned down in the discussion, which now fully acknowledges that conclusions cannot be drawn in such cases.

Additional changes

- A version of the chapter was previously submitted to *Journal of Experimental Biology*. Subsequently, some of the feedback from reviewers has been incorporated into the corrected version of the chapter.

- Table 1 gives more detail on maternal parameters and Table 2 shows how many animals provided data in each component of each experiment.
- Mixed effects models have been updated, and model results reported where previously there were none (e.g. offspring behavioural traits, cortisol). For offspring weight, behavioural traits, and swim distance models, sex was added as a fixed effect term instead of a random effect term. Early offspring size data were analysed and presented on the log scale. Post-hoc tests have also been applied where previously separate models were run to subsets of data.
- Analysis of body area was restricted to the first two weeks post-parturition as it was realised the course of discussion with the examiners that sexual differentiation of offspring would have made the data collected after that time difficult to interpret.
- Plots of body area for diet experiment have been changed to show all the datapoints instead of means and error bars for certain time windows, as in the previous version.
- The plot of growth rates (Fig. 4) now includes data from both GPS and diet experiments.

Chapter 3

Requested changes

- Content formerly contained in ‘Addendum 1’ has been fully integrated into the main chapter.
- Figures updated – removed titles above plots.
- Model results tables are presented for all key results, including in most cases parameter estimates of fixed effect terms and variance estimates of random effect terms. Sample size information is also given either in table legends or figure legends.
- Table 8 shows how many animals were used in each component of each experiment.
- Information on statistical models is expanded, and Table 9 shows the structure of all models, fixed and random terms, and exact sample sizes.

Additional changes

- ‘Summary’ section from previous version expanded into separate abstract and introduction.
- Use of experiment labels (Experiment 1, Experiment 2…) to make the story easier to follow.
- Streamlined statistical analyses: post-hoc tests of mixed effects models were used to compare specific groups, rather than the use of separate model fits as in the previous version. The only instance in which a separate model is used to compare specific groups is for larval thigmotaxis in experiment 2, for which some attempt at justification is presented in the text. The updated models also include the use of polynomial terms (quadratic, cubic) where the inclusion of these terms improved the model fit (reduced AIC).
- Experiment 2 larval thigmotaxis analysis expanded, now making use of data from earlier time points, rather than just the final minute of exposure as was the case before. The required analysis is more complex, but the result is more interesting.
- As the sample size for the stirring experiment was already somewhat limited, the dataset was adjusted to include only ‘paired’ samples – i.e. data from broods with both control and stirring samples available. I believed this to be justified because bias introduced from non-paired samples may have a stronger effect amongst a lower sample size. Filtering for pairs was not done for the alarm cortisol data as the sample size was considerably larger.
- For juvenile thigmotaxis in experiments 2 and 3, the response variable was changed from ‘% distance moved at periphery’ to ‘% time spent at periphery’, as the latter is a simpler, more common measure and is more equivalent to the response variables of the other two behavioural tests.

Chapter 4

Requested changes

- The introduction has been expanded to frame the chapter as an attempt to test the specific hypothesis that intergenerational effects of paternal stress revealed in Chapter 3 are mediated by spermatozoal RNA. To further develop this context, the introduction refers to previous rodent studies of paternal stress, findings of Chapter 3, and the details of mammalian and piscene spermatogenesis introduced in Chapter 1.
- Figures updated – removed titles above plots.

Additional changes

- A table (Table 1) has been added to show spermatozoal RNA yields.

Chapter 5

Requested Changes

- Discussion of the findings in relation to DOHaD has been expanded.
- Discussion of potential mechanisms to explain intergenerational effects has been expanded. Specifically, a section has been added which discusses evidence for the role of extracellular vesicles (EVs) in the modification of germ cells, citing two recent studies suggested by the examiners.
- Content previously included as part of 'Chapter 2, Addendum 2' describing a preliminary gene expression experiment has been integrated into a section on proposed future work.

A brief note on Appendix 1

It was suggested by the examiners that the contents of Appendix 1 be moved to a main results chapter. However, given that the manuscript was framed in the context of environmental toxicology rather than epigenetics and parental effects, it is contextually removed from the rest of the thesis, and thus I felt it best left as an appendix. It has been updated since the version included in the original thesis to include additional background literature and some additional data which was not originally included.

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