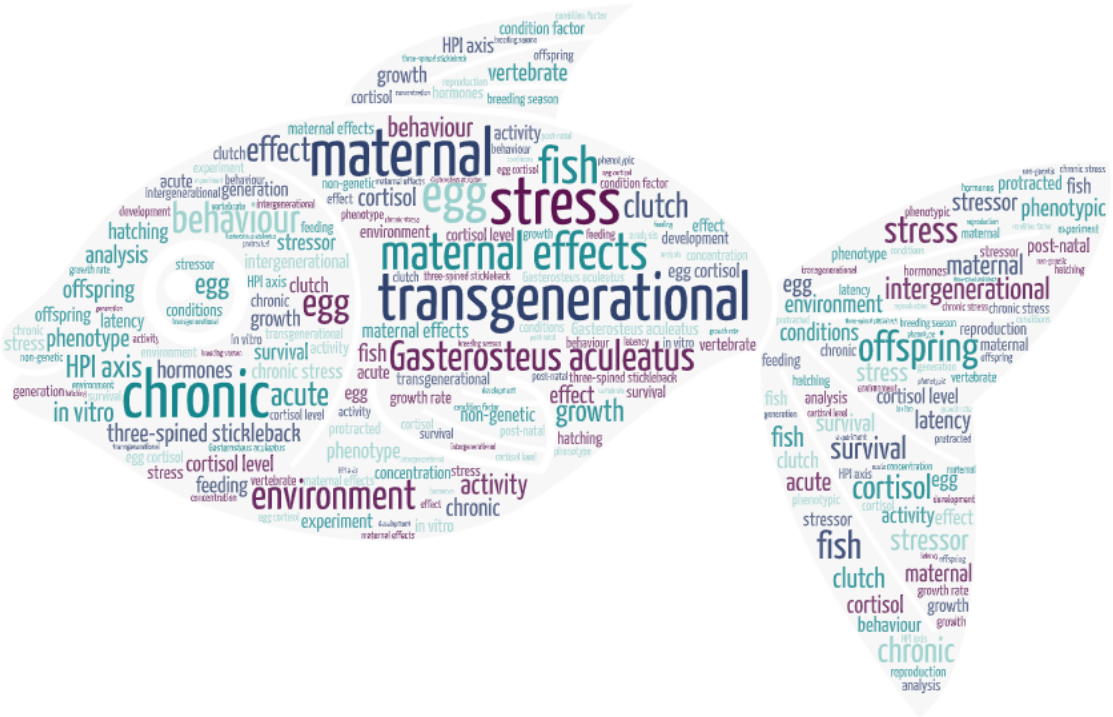
TRANSGENERATIONAL EFFECTS OF CHRONIC ENVIRONMENTAL STRESS



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General abstract

In this thesis I explore the topic of maternal effects, and namely the effects of maternal stress on various aspects of offspring phenotype. More specifically, I examine how chronically stressful environmental conditions experienced by females in the period leading up to egg production and spawning influence their reproductive strategies, the levels of glucocorticoid hormones deposited in their eggs and the phenotype of their offspring. I also investigate whether the potential effects interact with seasonal patterns in maternal provisioning and whether they span more than one generation. The results of this longitudinal study performed on wild three-spined sticklebacks (*Gasterosteus aculeatus*) in controlled laboratory conditions are presented as a series of chapters, each exploring different aspect of maternal stress effects.

Exposure to stressful conditions can have a profound effect on animal phenotype, for example in terms of their stress physiology, behaviour and overall body condition. In Chapter 2, I demonstrate that chronic stress due to a combination of environmental and husbandry stressors did not have a physiological effect on female sticklebacks in terms of their baseline cortisol level, hormonal response to an acute stressor nor their body condition. It did however influence their behaviour, with immediate effects on activity levels and a longer-term effect on feeding behaviour.

Females in stressful environments may attempt to shift their reproductive strategies to increase their fitness in the prevailing conditions. These adjustments may vary depending on the seasonal fluctuations in reproductive investment and the timing of stress exposure. This is the topic of Chapter 3, where I show that various egg/offspring characteristics are altered by maternal stress exposure. I also demonstrate that some of these effects may vary with the position in the sequence of clutches produced by a female across the breeding season. However, since neither stress-exposed females nor their eggs showed increased cortisol levels, any observed effects are not likely to be driven by maternally-derived glucocorticoids.

In addition to the effects on pre-natal developmental trajectories, maternal stress can influence various aspects of offspring post-natal phenotype. This is often interpreted as adaptive maternal programming, where females pre-program their offspring to better cope with the anticipated environmental

conditions. However, with protracted stress exposure the adaptive potential of maternal programming is less clear, particularly in combination with seasonal maternal adjustments to offspring phenotype. In Chapter 4, I provide evidence that chronic maternal stress may reshape inter-clutch patterns of offspring survival, but whether this effect is adaptive or maladaptive depends on offspring age. Regardless of maternal treatment, there was also inter-clutch difference in growth rate. However, as with survival, the benefit of this seasonal maternal adjustment was age-dependent. In Chapter 5, I demonstrate that offspring behavioural phenotype and response to stressors may be influenced by chronically stressful maternal conditions. Stress-exposed mothers produced offspring that showed reduced similarity of behaviour between related individuals, which may be an example of a maternal strategy to increase fitness through bet-hedging in an unpredictable environment. I also provide suggestive evidence that maternal stress interacts with offspring position in the sequence of clutches in shaping the offspring's hormonal response to acute stressors.

In Chapter 6, I examine whether any observed effects persist further down the maternal line or whether any new effects manifest themselves in later generations. My results indicate that chronically stressful conditions experienced by females may affect reproductive strategies of their daughters and pre-natal developmental trajectories of their grandoffspring, and that this effect may depend on the order of clutches produced by these females. Despite being complex and highly context-dependent, the observed relationships suggest that stress-exposed females may indirectly influence reproductive investment of their daughters in an attempt to increase their fitness.

The significance of my results is discussed in detail in Chapter 7, but overall this thesis provides evidence that protracted maternal exposure to stressful conditions can have far-ranging consequences for various aspects of an offspring's phenotype and that these effects are not always straightforward and may be modified by seasonal patterns of maternal investment. Furthermore, I demonstrate that stress experienced within a single generation can influence subsequent generations that do not experience the same stressful conditions.

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Acknowledgements

"*I feel a very unusual sensation – if it is not indigestion, it must be gratitude*."

Benjamin Disraeli

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Author's Declaration

I declare that this thesis is the result of my own work conducted between October 2015 and September 2019. No part of this work has been submitted for any other degree or qualification, at the University of Glasgow or any other institution.

The work is based on individual research carried out by myself. Antreas Aristeidou, Savvas Ioannou and Åsa Lind assisted with *in vitro* breeding and collection of data on female behaviour, cortisol level, condition and reproductive strategy (Chapters 2 and 3), and on offspring growth and survival (Chapter 4). Antreas Aristeidou, John Gibson and Maria Papaevripidou helped with *in vitro* breeding and collection of data on transgenerational stress effects, contributing to Chapter 6.

This study complied with the Animals (Scientific Procedures) Act 1986 (Project license no. P89482164 held by N.B. Metcalfe, personal license no. I715DABF6 held by A. Magierecka) and was approved by the University of Glasgow Animal Welfare and Ethical Review Board.

Agnieszka Magierecka

Date: 11/10/2019

Chapter 1: General introduction

Environmental stress, stress response and its adaptive potential

The influence of stress on growth, survival, life-history traits and behaviour of animals has been the focus of scientific research ever since the ground breaking work of Hans Selye, who described, for the first time, a range of physiological responses to acute stressors (Selye, 1936). Stemming from this work is the field of stress ecology, which provides a link between the environmental conditions experienced by organisms and various aspects of their performance. Due to the diversity of factors involved in animal response to adverse environmental conditions, defining stress has been a subject of an ongoing debate (Romero, 2004, McEwen, 2005, Johnstone *et al.*, 2012), and has once been described as a task belonging to someone who "has an enormous ego, is immeasurably stupid, or is totally mad" (Levine, 1985, p.51). Nevertheless, stress may be generally referred to as a situation in which adverse aspects of the environment (stressors) lead to the disturbance of organism's internal balance, causing an animal to adjust its behaviour and/or physiology in order to restore homeostasis (McEwen and Wingfield, 2003, Romero, 2004).

The environment is rarely invariable and various abiotic and biotic factors may change on a short- and/or long-term scale, leading for example to perceived deterioration of environmental conditions and the state of stress. These factors may include temperature, light levels, food abundance, predator pressure, parasites and pathogens. In addition, animals may be subject to changes in their social environment, for example to fluctuations in population density and composition. Moreover, climate change and human-induced environmental change (e.g. light, noise and chemical pollution, habitat modification) are additional sources of stress for wild animals. Environmental and anthropogenic sources of stress in the wild have been reviewed in detail by such as Baker *et al.* (2013) and Dantzer *et al.* (2014). Animals living in captivity can experience additional stressors related to capture, handling, restraint, overcrowding etc. (Portz *et al.*, 2006, Morgan and Tromborg, 2007). All of these factors, acting individually or synergistically, have a potential to disrupt an organism's internal

balance and induce a stress response (Bijlsma and Loeschcke, 2005, Holmstrup *et al.*, 2010, Baker *et al.*, 2013).

In higher vertebrates, the stress response is mediated by the hypothalamic-pituitary-adrenal (HPA axis; Figure 1.1). The main elements of the HPA (stress) axis are the hypothalamus and pituitary gland in the brain, and adrenal glands located above the kidneys (Tsigos and Chrousos, 2002, Herman *et al.*, 2016). In fish, interrenal cells perform the same function as adrenal glands in other vertebrates and thus their stress axis is referred to as the hypothalamic-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997). Exposure to a perceived stressful stimulus leads to activation of the stress axis through secretion of corticotropin-releasing hormone (CRH) from the hypothalamus. CRH acts upon the pituitary gland, stimulating the release of adrenocorticotropic hormone (ACTH), which influences the adrenal glands (or interrenal cells in fish) and ultimately results in secretion of glucocorticoid (GC) hormones (Sapolsky *et al.*, 2000, Herman *et al.*, 2016). GCs (e.g. cortisol and corticosterone) are transported through the systemic circulatory system to various target tissues, where they interact with specific receptors through which they exert their effects (Tsigos and Chrousos, 2002, Herman *et al.*, 2016). Activation of the HPA/HPI axis, and the ensuing hormonal cascade, is a generalised, or primary, response of organisms to stressful stimuli. The secondary stress response involves further biochemical and physiological adjustments. These include increased breakdown of glycogen in liver and enhanced transport of glucose to muscles, cardiovascular and immune activation, release of stress proteins (e.g. heat shock proteins) and downregulation of sex hormone production (Sapolsky *et al.*, 2000, Johnstone *et al.*, 2012) Thus, short-term exposure to an acute stressor (e.g. predator encounter, capture, short-term restraint) is thought to be adaptive as it initiates a physiological response for coping with noxious stimuli and facilitates recovery (Sapolsky *et al.*, 2000, Romero, 2004, Herman *et al.*, 2016). If the stressor is transient, feedback loops associated with the stress axis downregulate the production of hormones once the stressor has ceased and homeostasis is restored (Romero, 2004).

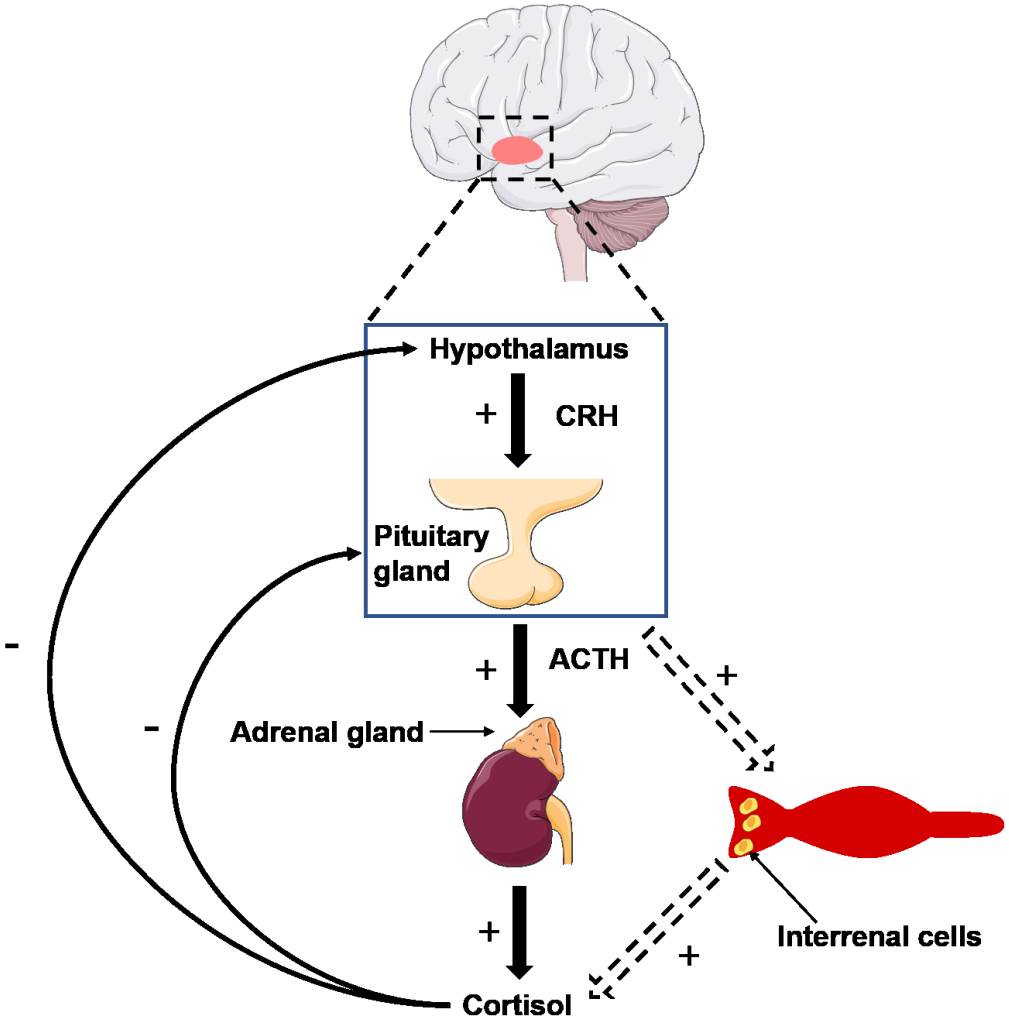

Figure 1.1 Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis in vertebrates. Exposure to stressful stimuli triggers production of corticotropin-releasing hormone (CRH) in the hypothalamus. CRH acts upon the pituitary gland stimulating release of adrenocorticotropic hormone (ACTH), which in higher vertebrates promotes synthesis of glucocorticoids (e.g. cortisol) by the adrenal glands. Increasing levels of cortisol exert negative feedback on ACTH and CRH production. The dashed arrows represent the alternative pathway (hypothalamic-pituitary-interrenal, HPI, axis) in fish, where ACTH acts upon interrenal cells in the head kidney.

However, if an animal is subjected to unfavourable environmental conditions over the long term, various cellular-level adjustments may have far-ranging consequences for the entire organism, resulting in a tertiary stress response and adjustments to morphology, physiology and behaviour (Romero, 2004, Johnstone *et al.*, 2012, Sopinka *et al.*, 2016a). As opposed to an acute stress response, which has a positive effect and facilitates coping with stressors, chronic activation of the stress axis is generally considered maladaptive (Sapolsky *et al.*, 2000, McEwen and Wingfield, 2003, Dantzer *et al.*, 2014). The stress response and the associated biochemical and physiological adjustments are energetically expensive (Picard *et al.*, 2018) and thus tend to divert energy resources away from other functions, which may result for example in the suppression of growth

(Müller *et al.*, 2009, Midwood *et al.*, 2014) and poorer body condition (van de Pol *et al.*, 2017) if the exposure to stressors is protracted. Exposure to chronically stressful conditions may also result in suppression of the response to subsequent acute stressors (Rich and Romero, 2005, Jeffrey *et al.*, 2014) and therefore reduce the adaptive effect of the primary and secondary stress response. In addition, chronic stress may alter various aspects of an animal's behaviour (Bliley and Woodley, 2012, Lawrence *et al.*, 2018b) and cognitive function (Sandi, 2013, Boogert *et al.*, 2018, van der Kooij *et al.*, 2018), and lead to shifts in its life history and fitness (Ricklefs and Wikelski, 2002, Bonier *et al.*, 2009, Crespi *et al.*, 2013). There is also increasing evidence that effects of chronic stress exposure in one generation can have far-ranging consequences for future generations, for example through maternal effects.

Effects of maternal stress on offspring phenotype

Alongside genetic and environmental factors, non-genetic parental influences play an important role in shaping of the offspring phenotype and determining their performance. Maternal effects, broadly defined as any non-genetic maternal contribution to offspring phenotype, are an important tool in translating maternal condition into various aspects of offspring morphology, physiology and behaviour through intergenerational phenotypic plasticity (Bernardo, 1996, Mousseau and Fox, 1998, Wolf and Wade, 2009). Therefore, if a female experiences stressful environmental conditions, this may have a profound effect on her offspring. There are many non-genetic mechanisms through which maternal stress can be translated into the phenotype of their offspring, including alteration of DNA methylation patterns (Rubenstein *et al.*, 2016), transfer of maternal mRNAs (Colson *et al.*, 2019) and differential allocation of nutrients in developing eggs or embryos (Ensminger *et al.*, 2018). However, perhaps the most important mechanism through which stress-exposed mothers influence the phenotype and performance of their offspring are hormonally-mediated maternal effects.

Maternal androgens have long been considered an important factor for offspring development and post-natal phenotype (Eising *et al.*, 2001, Groothuis *et al.*, 2005, Uller *et al.*, 2007), but the same is also true for glucocorticoids. Typically, exposure of females to stressful environmental conditions results in activation of their HPA/HPI axis and increased production of GCs. If this occurs in the period

leading up to egg production and spawning, an increase in egg/embryo GC level and subsequent developmental and phenotypic alterations may be observed. A classic example is an experiment on Japanese quail (scientific names of all species are given in Appendix 1), where females were implanted with corticosterone (CORT) to mimic stress exposure. CORT-implanted females produced eggs with a greater concentration of CORT in the yolk, and the resulting offspring exhibited a reduced growth rate and increased activity of the stress axis (Hayward and Wingfield, 2004). Maternally-derived glucocorticoids can affect a whole suite of offspring phenotypic traits, ranging from developmental trajectories, post-natal survival and growth (Love *et al.*, 2005b, Gagliano and McCormick, 2009, Dantzer *et al.*, 2013), through stress responsiveness (Bian *et al.*, 2015, Eriksen *et al.*, 2015, Weber *et al.*, 2018) to various aspects of behaviour and cognitive ability (Love *et al.*, 2005a, Eaton *et al.*, 2015, Ensminger *et al.*, 2018).

Despite the mounting evidence that maternal stress can affect physiology and behaviour of offspring, there is a lack of agreement as to whether these maternal effects are adaptive or maladaptive for the offspring during their developmental stages and in later life. The adaptive potential of maternal stress is likely to be highly context-dependent and reliant on the interplay between environment (maternal and offspring), genetic inheritance, nature and duration of the stressful stimuli etc. (Marshall and Uller, 2007, Sheriff and Love, 2013a, Love *et al.*, 2013). In an adaptive maternal effect scenario, stress-exposed females adjust the phenotype of their offspring to increase their survival potential in the anticipated stressful environment (Dantzer *et al.*, 2013, Uller *et al.*, 2013). The same adjustments may be maladaptive and result in reduced offspring survival and fitness, for example when the offspring environment does not match that of a mother or if it is unpredictable or highly variable (Marshall and Uller, 2007, Hoyle and Ezard, 2012, Sheriff *et al.*, 2018). Maternal stress may also have negative consequences for the offspring if it results from passive transfer of hormones rather than an active maternal adjustment of their concentration in eggs or embryos (Heath and Blouw, 1998). As opposed to acute stress, which tends to initiate an adaptive response, chronic stress can have a more deleterious effect, particularly when it is unpredictable and thus preventing habituation (Sapolsky *et al.*, 2000, Romero, 2004); it is therefore possible that the adaptive potential of maternal stress may be highly dependent

on whether the stressful stimuli are transient or protracted. In addition, there is emerging evidence of metabolic processes in the developing embryos that result in removal of maternally-derived glucocorticoids and thus may protect offspring from potential adverse effects of maternal stress (Paitz *et al.*, 2016, Carter *et al.*, 2018).

In addition to immediate effects of maternal stress on various aspects of offspring phenotype, the consequences of the environmental conditions experienced by females may persist or even manifest themselves in the subsequent generations (Burton and Metcalfe, 2014, Khan *et al.*, 2016a, Shama *et al.*, 2016). These are termed transgenerational effects if found in the F2 or later generations, since these individuals were not directly exposed to the stressor (unlike the F1 generation, which was exposed while at the germ cell stage in the parental, F0, generation (Knudsen *et al.*, 2018)). These longer-term effects may be particularly pronounced if maternal stress exposure alters offspring life history traits and reproductive strategies (Sorci and Clobert, 1995, Connor *et al.*, 2012, Dupont *et al.*, 2012), for example in terms of the quantity of eggs, which tends to be directly related to the quality of the offspring (Naguib and Gil, 2005). The direction and magnitude of such transgenerational effects is likely to be dependent on the stability of the environment and the extent of grandmaternal-grandoffspring environment matching, particularly in the context of anticipatory phenotype adjustments (Herman *et al.*, 2014, Engqvist and Reinhold, 2016). Thus if the environment is stable across the generations, there may be an accumulation of phenotypic effects (Shama and Wegner, 2014, Le Roy *et al.*, 2017), but if grandoffspring don't experience the same conditions as their grandmothers, various compensatory mechanisms may offset potential phenotypic adjustments (Shama *et al.*, 2016).

Current issues and open questions

Despite the growing body of research on the effects of stress on organisms and subsequent inter- and transgenerational effects, there remain many issues that still hinder our understanding of the mechanisms and outcomes of exposure to environmental stressors. A review of the field of stress ecology shows a strong taxonomic bias in the available data, with a large proportion of research being conducted on birds (Breuner *et al.*, 2008) and salmonid fishes (Van Weerd and Komen, 1998, Sopinka *et al.*, 2016a). The biology and ecology of birds and

salmonids differs considerably from that of other taxa, including non-salmonid iteroparous fishes (which, for instance, generally produce much smaller eggs than salmonids); therefore more research is required to disentangle the effects of maternal stress in these organisms. Where multigenerational effects of environmental conditions are taken into account, these studies are performed mostly on insect models, with relatively few examples from vertebrates. This is mainly due to the ease of keeping and breeding invertebrates and the short generation time, which allows for measuring the effects across many generations over a relatively short period of time (Andre *et al.*, 1989). However, as with the differences between vertebrate taxa, the effects in invertebrates may be profoundly different to those in vertebrates, particularly due to their short life span and rapid succession of generations.

In addition to the taxonomic bias, there is also a bias in methodology used for studying the effects of maternal environmental stress on offspring phenotype and performance. The research to date has relied heavily on measuring the effects of acute stressors; one of the reasons behind this is the relative ease with which acute stress responses can be induced and quantified, for example through exposure to a predator, capture, territorial challenge or glucocorticoid injection (Breuner *et al.*, 2008). There are relatively few attempts to quantify the effects of chronic unpredictable maternal stress, which could provide an insight into the effects that environmental change (and the potential for increased environmental stress) may have on wild populations; chronic maternal stress due to a combination of environmental and husbandry stressors may also have negative consequences for captive populations. Many experimental studies use GC implants to mimic the exposure to chronically stressful environmental conditions in females and induce the phenotypic effects in their offspring. Similarly, exposure of eggs to exogenous glucocorticoids through immersion or injection is often employed as a proxy of maternal stress. However, both of these methods have serious shortcomings, which may render them of limited value as a proxy of the processes occurring in organisms subjected to environmental stressors, since these may be more complex in nature. Even if the GC implants contain biologically relevant concentration of hormones, the rate of hormone release from these implants may not be constant (Crossin *et al.*, 2016), and elevated GC rates early in the experiment may alter receptor sensitivity (Dufty *et al.*, 2002), resulting in spurious results later on. Moreover, the

implantation process itself can have an intergenerational effect, even in the absence of hormones (Hoogenboom *et al.*, 2011). In terms of subjecting eggs to exogenous hormone treatment, this procedure reflects potential phenotypic changes due to the GCs alone, not taking into account other stress-induced maternal factors that may play an important role in shaping of the offspring phenotype, such as androgens (Henriksen *et al.*, 2011), thyroid hormones (Ruuskanen and Hsu, 2018) and nutrients (Ensminger *et al.*, 2018). An additional drawback of experimental approaches utilising exogenous hormones is the complexity of the processes involved in transfer of maternal hormones, which may involve both maternal and embryonic attempts to regulate the levels of GCs reaching the developing eggs or embryos (Faught *et al.*, 2016, Paitz *et al.*, 2016, Carter *et al.*, 2018), but which may be absent where exogenous GCs are used.

An important assumption in the studies of inter- and transgenerational stress effects is that the females subjected to stressful stimuli respond by mounting a stress response. The concentration of glucocorticoid hormones in plasma or tissue (or in holding water in case of some studies on fish) is commonly used as a measure of stress as it is thought to reflect the level of HPA/HPI axis activation following exposure to stressful stimulus (Dantzer *et al.*, 2014, Sopinka *et al.*, 2016a). Whilst it is likely to be true for transient and acute stressors, it is not clear whether this method is adequate to assess the response to stressful environmental conditions experienced on a longer-term scale. Protracted exposure to stressors may lead to exhaustion of the HPA/HPI axis and cessation of the GC synthesis (Romero, 2004, Madaro *et al.*, 2015). However, even in the absence of primary stress response (a situation which may be confused with habituation to the stressors), changes may still be observed at a whole organism level, for example in terms of body condition and behaviour (Dantzer *et al.*, 2014, Sopinka *et al.*, 2016a, van de Pol *et al.*, 2017). In addition, relying on GC measures alone for long-term or longitudinal experiments may be impractical or impossible as some of the GC sampling methods may be stressful or even destructive. Nonetheless, few studies employ multiple, non-GC based measures of stress.

Furthermore, whilst the effects of maternal stress on various aspects of offspring phenotype and performance have been previously demonstrated in a wide range of vertebrate taxa (Hayward and Wingfield, 2004, Green, 2008, Bian *et al.*, 2015, Ensminger *et al.*, 2018), an important question that remains unanswered is

that of how maternal stress affects offspring from different reproductive attempts if the stressful conditions persist throughout the entire breeding season, or the entire reproductive lifespan of a female. Since the stressful stimuli may interact with other factors, such as age and body condition of a female, it is reasonable to expect that chronic maternal stress in these circumstances will have a differential effect on offspring from successive broods, with two possible scenarios being accumulation or lessening of the maternal stress-induced phenotypic effects. However, to the best of my knowledge no attempts have been made to quantify such inter-brood variations in response to maternal exposure to chronically stressful and unpredictable environment.

In an attempt to shed some light on these issues, I performed a longitudinal laboratory-based experiment in which three-spined sticklebacks (*Gasterosteus aculeatus*) were subjected to a period of unpredictable chronic stress due to a combination of environmental and husbandry stressors in the period leading up to egg production and spawning. I then measured a range of offspring and grandoffspring phenotypic traits in order to disentangle the effects of chronic maternal stress on phenotype and performance of offspring from different clutches produced across the breeding season. Figure 1.2 shows an overview of the longitudinal experiment with reference to the specific chapters of the thesis.

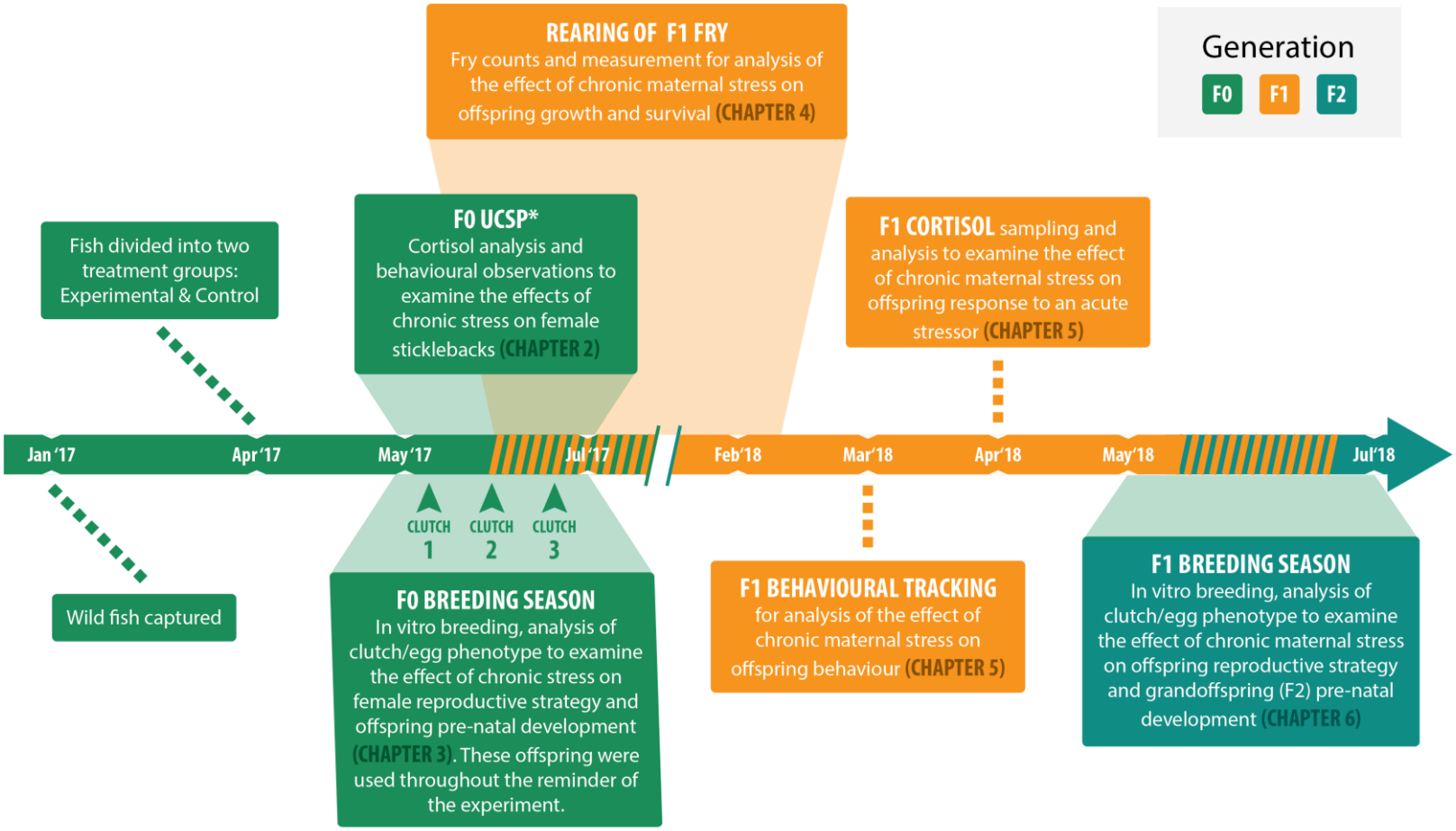

Figure 1.2 Overview and timeline of the longitudinal experiment examining inter- and transgenerational effects of chronic environmental stress in three-spined sticklebacks. During the 2017 breeding season the F0 females produced up to three clutches, indicated here as Clutch 1, 2 and 3. The dashed areas represent periods during which the generations overlapped. * UCSP indicates Unpredictable Chronic Stress Protocol.

Study species

The three-spined stickleback (Figure 1.3) is a small teleost fish, widely distributed across the Northern Hemisphere and present in both Atlantic and Pacific Ocean around the coasts of North America, Europe and Asia (McKinnon and Rundle, 2002). Whilst marine in their origin, three-spined sticklebacks colonised freshwater habitats and can be found mostly in shallow area of streams, rivers and lakes throughout their range; thus currently three main forms can be distinguished: marine, freshwater and anadromous (Wootton, 1976, Bell and Foster, 1994). In general, they are cryptic in colouration, with armour plating and three distinctive dorsal spines; however, the colouration and the extent of the plating may vary in different populations (Bell and Foster, 1994, Kitano *et al.*, 2007). During the reproductive season, mature males adopt nuptial colouration consisting of red throat and blue eyes (Wootton, 1976, Franco-Belussi *et al.*, 2018). Parental care in three-spined sticklebacks is limited to males, who use plant material and spiggin (protein synthesised in kidneys) to build a nest (Wootton, 1976, Jakobsson *et al.*, 1999). Once a female deposits eggs in the nest, they are fertilised by a male, who then defends the nest, cleans and aerates the eggs and guards fry for approximately two weeks after hatching (Wootton, 1976, Whoriskey and FitzGerald, 1994). Female reproductive strategies differ between different forms and populations (Baker *et al.*, 2015). In general, three-spined sticklebacks are iteroparous, meaning that they produce multiple clutches during their reproductive lifespan. Some females produce offspring over several reproductive seasons, but many freshwater populations are annual, i.e. produce multiple clutches within a single breeding season, usually in the first year of life; the majority of laboratory sticklebacks also breed in the first year (Lee *et al.*, 2012, Baker *et al.*, 2015). Depending on size, diet and condition, as well as on the duration of the breeding season, wild females can produce up to nine clutches in a breeding season, with laboratory females often showing considerably lower seasonal reproductive output (Whoriskey and FitzGerald, 1994, Baker *et al.*, 2015).

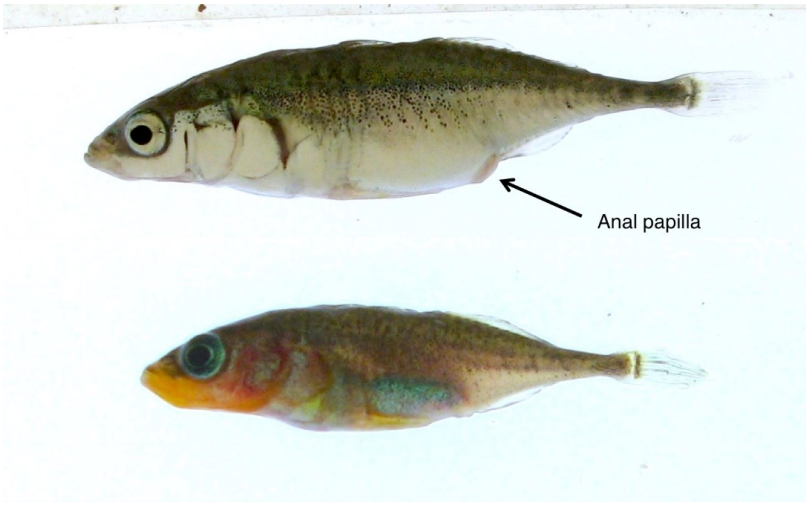

Figure 1.3 Mature female (top) and male (bottom) three-spined stickleback (*Gasterosteus aculeatus*). The female shows signs of readiness to spawn – expanded abdomen and distended anal papilla. The male has adopted a nuptial colouration, as indicated by the red throat and blue eye.

There are multiple factors that make three-spined stickleback an ideal species to experimentally assess inter- and transgenerational effects of chronic stress. They are relatively easy to keep in laboratory conditions due to their small size (and hence low space requirement), varied diet and resistance to variations in water temperature and other environmental parameters (Heng *et al.*, 2016); as a result, sticklebacks have been widely used as a model in fields ranging from neuroscience (Norton and Gutierrez, 2019) to toxicology (Pottinger *et al.*, 2002, Katsiadaki *et al.*, 2007) and the effects of climate change (Shama, 2015). Since females do not provide maternal care, any phenotypic effects in offspring are independent of care strategies, and they can be further controlled for by using *in vitro* fertilisation (Heng *et al.*, 2016). Female reproductive decisions (whether to mature and reproduce or postpone reproduction) are generally established in the winter preceding the breeding season (Sokolowska and Kulczykowska, 2006), therefore exposure to chronic stress during the reproductive period is unlikely to prevent development and maturation of ova. If certain conditions (e.g. diet) remain constant, the reproductive output of female sticklebacks tends to be consistent across the breeding season (Wootton and Fletcher, 2009, Baker *et al.*, 2015), but changes to various environmental parameters, such as temperature, may result in shifts in reproductive strategy (Kim *et al.*, 2017). As a result, potential differences in clutch characteristics and offspring phenotype may be pinned down to the effects of stress with a high degree of certainty. In addition, three-spined sticklebacks are synchronous spawners, with all ova developing simultaneously and at a rapid rate (Wallace and Selman, 1979). Therefore any

potential effects of chronic maternal stress exposure are likely to be the same in all offspring from a given clutch, minimising the confounding effect of inter-individual differences in the degree of maternal stress-driven phenotypical change.

Various methods have been established to assess the effects of stress in three-spined sticklebacks, including a range of physiological (Pottinger *et al.*, 2002, O'Connor *et al.*, 2011) and behavioural (Bell *et al.*, 2010, Feng *et al.*, 2015) indices, as well as non-invasive and minimally stressful methods of cortisol sampling developed and validated for sticklebacks and allowing for multiple measurements of the same individuals over a period of time (Sebire *et al.*, 2007, Sebire *et al.*, 2009). These methods, combined with the specific ecology and reproductive biology of three-spined sticklebacks, make this species of great value for experimental studies of inter-clutch differences in offspring phenotypic response to chronic maternal stress.

Aims of the thesis

The main aim of this thesis is to explore the effects of maternal exposure to chronically stressful and unpredictable environmental conditions on various aspects of phenotype and performance of offspring and grandoffspring from successive clutches produced by females over a single breeding season. This is attempted over five data chapters (thesis chapters 2-6). In Chapter 2, I examine primary and tertiary response of female three-spined sticklebacks to unpredictable chronic stress protocol (UCSP), using a range of physiological and behavioural indices. In Chapter 3, I use *in vitro* breeding to scrutinise the effect that exposure to a chronically stressful environment has on female reproductive strategy across the breeding season and on offspring pre-natal developmental trajectories. Chapter 4 uses a longitudinal approach based on regular measurements of non-stressed fry/juveniles in the first six months of their life to examine the effects of chronic maternal stress on offspring early-life survival and growth. I also examine whether the potential effect differs across the sequence of clutches produced over the breeding season. In Chapter 5, juvenile offspring of stressed females are subjected to an acute stressor to determine if maternal stress exposure alters offspring stress physiology in terms of their peak cortisol level. I also perform a range of behavioural observations in a controlled environment to detect potential differences in behaviour between the offspring

of stress-exposed and non-stressed females. Chapter 6 examines the effect of stressful and unpredictable environment experienced by females from the F0 generation on reproductive effort of their offspring (F1 generation) and pre-natal developmental trajectories of their grandoffspring (F2 generation) in the context where the offspring do not experience the same stressful conditions as their mothers. The final chapter (Chapter 7) is a general discussion of the results, which puts them in the perspective of current research on the effects of maternal stress.

Chapter 2: The effect of chronic stress exposure on female three-spined sticklebacks (*Gasterosteus aculeatus*)

Abstract

Animals are often exposed to changes in their environment that may initially be perceived as stressful, leading to an acute stress response, but to which they can eventually habituate. However, if habituation to stressors is prevented, e.g. due to the stressors being diverse and unpredictable in the timing and sequence of their appearance, a state of chronic stress may ensue. Organisms exposed to chronic stress may show altered baseline levels of glucocorticoid hormones and change in behavioural patterns (e.g. activity and feeding behaviour), but the exact phenotypic effects of chronically stressful environments remain poorly understood.

In this study, I exposed three-spined sticklebacks to an unpredictable sequence of brief stressors over a protracted period and measured their response in terms of cortisol levels, body condition indices and behavioural patterns. I quantified their activity and feeding behaviour in two contexts: while the stressors were applied and during the resting period between stressors.

I did not observe a change in baseline cortisol levels following a period of chronic stress nor any effect on cortisol levels in response to an acute stressor. In addition, body condition indices were unaffected. The exposure of fish to chronic stress led to a decline in latency to feed during the resting periods, indicative of an anticipation of future stress exposure. I observed an increase in activity levels of the stress-exposed fish, but only during the presentation of the stressors. Organismal response to protracted periods of stress is energetically expensive, thus my result may indicate a trade-off between energy-demanding activities in fish subjected to unpredictable chronic stress.

Introduction

Both wild and captive animals face a multitude of environmental stressors throughout their lifetime. These stressors may include changes in temperature, light levels and food abundance, predator pressure, exposure to pollutants etc., working individually or synergistically, and eliciting an organismal response. This initial, generalised stress response is associated with the release of glucocorticoid (GC) hormones (Sapolsky *et al.*, 2000). Following the primary stress response, a range of biochemical and physiological adjustments occur at a cellular level, leading to a secondary stress response, including changes to metabolism (Sapolsky *et al.*, 2000, Barton, 2002). Ultimately, these cellular-level adjustments may lead to organismal-level changes (tertiary stress response) affecting a wide range of morphological, physiological and behavioural features, such as structure and function of various organs (Harper and Wolf, 2009, Jones *et al.*, 2018), growth (Müller *et al.*, 2009, Tsalafouta *et al.*, 2015), mortality (Nagrodski *et al.*, 2013, Razzoli *et al.*, 2018) and cognitive function (Piato *et al.*, 2011, Boogert *et al.*, 2018).

In vertebrates the fundamental system underlying the response to environmental stress is activation of the hypothalamic-pituitary-adrenal (HPA) or (depending on the taxon) the hypothalamic-pituitary-interrenal (HPI) axis. This ultimately results in secretion of glucocorticoid hormones as a generalised stress response (Sapolsky *et al.*, 2000, Johnstone *et al.*, 2012), with the action of these hormones mediated by glucocorticoid receptors in target tissues (Sapolsky *et al.*, 2000, Alderman *et al.*, 2012, Senft *et al.*, 2016). Primary and secondary stress responses are often associated with acute stress, or early stages of stress, which initiate a physiological response for adaptive coping with noxious stimuli (Barton, 2002, Busch and Hayward, 2009). Once the stressor is removed from the environment, homeostasis is restored through negative feedback on the HPA/HPI axis. However, if the stressor persists beyond the initial hormone-induced response, and particularly if its nature is unpredictable and therefore prevents habituation, it may lead to a tertiary stress response and a state of chronic stress, which has been shown to have a more deleterious effect on organisms (Barton, 2002, Romero, 2004, Busch and Hayward, 2009).

There is limited evidence, stemming mostly from studies on aquatic vertebrates, that environmental stressors can act synergistically, hindering the ability of

animals to mount an adaptive response. For example, goldfish exposed to a combination of chemical and physical stressors showed attenuated cortisol secretion, reduced antioxidant defenses and overall disturbance in energy metabolism (Gandar *et al.*, 2016). There is also increasing evidence that routine animal husbandry practices, such as capture, confinement and tagging, can lead to elevation of stress hormone levels (Portz *et al.*, 2006, Morgan and Tromborg, 2007, Furtbauer *et al.*, 2015). Protracted chronic stress due to a combination of environmental and recurring husbandry stressors has therefore negative consequences for animals, particularly those held in intensive agriculture or aquaculture systems. This has been demonstrated in Atlantic salmon, where fish challenged with a range of random stressors (social, visual, temperature etc.) for a prolonged period of time had a reduced response to subsequent acute stressors (Madaro *et al.*, 2015). Similar attenuation of the acute stress response was observed in European starlings subjected to chronic stress due to a combination of chemical (dexamethasone) and physical (restraint) stressors (Rich and Romero, 2005). Zebrafish challenged with an unpredictable chronic stress protocol demonstrated a range of altered behaviours, including reduced cognitive function and increased levels of anxiety (Piato *et al.*, 2011). These behavioural changes may result from alteration of certain brain structures following prolonged stress exposure, as observed e.g. in chickens (Gualtieri *et al.*, 2019).

Despite the extensive evidence of the effects of stress on various aspects of an animal's phenotype, the effects of chronic stress remain poorly understood. The majority of studies addressing the effects of chronic stress on animals tend to use single, repeated stressors (e.g. confinement) or employ exogenous glucocorticoids such as cortisol (as a feed supplement or implants) as a proxy for chronic stress (Van Weerd and Komen, 1998, Sopinka *et al.*, 2015b). Particularly in the case of implants, it is unclear whether the release of hormones takes place at a constant and biologically relevant rate throughout the experimental period (Crossin *et al.*, 2016). It has also been previously shown that implantation itself may not be without an effect (Lapin, 1995, Hoogenboom *et al.*, 2011), so making the protocol of questionable relevance to the stressors normally encountered by the animal. Few studies employ multiple stressors to elicit the effects of chronic stress, and the ones that have been used have tended to be relatively severe (Piato *et al.*, 2011, Madaro *et al.*, 2015), whilst many

ecologically-relevant stressors are likely to be considerably milder. Low-intensity stressors (e.g. changes in light intensity and novel objects in the tanks) were employed in a study of the effects of unpredictable, chronic stress on the development of European sea bass, and were shown to lead to increased cortisol release rates and lower performance of juvenile fish (Tsalafouta *et al.*, 2015). Similarly, chronic exposure of chickens to a range of mild husbandry and social stressors resulted in altered development of brain regions responsible for behaviour (Gualtieri *et al.*, 2019). The question however largely remains of how multiple, low-intensity stressors applied over a prolonged period of time affect various aspects of the animal's phenotype.

In this study, I address this open question by simulating low intensity, unpredictable chronic environmental stress and investigate its effect on three-spined sticklebacks. The stressors were applied during the breeding season since this is a period crucial for the lifetime fitness of fish, and because it may also influence the phenotype of their offspring (Schreck *et al.*, 2001). Firstly, I examined the primary (hormonal) response of breeding females to chronic stressors by measuring changes in baseline cortisol levels, and the potential modulation of the HPI axis by looking at cortisol levels following exposure to a subsequent acute stressor. Secondly, I explored whole-animal level changes (the tertiary stress response) after a period of chronic stress by assessing differences in in body condition indices and behaviour between stress-exposed and stress-unexposed fish. Due to the high variation in hormonal response of vertebrates to chronic stress (Dickens and Romero, 2013) it was impossible to make predictions as to the effect of chronic stress on baseline cortisol levels and the response to a subsequent acute stressor. However, I anticipated that stress-exposed fish would to be in poorer body condition than non-exposed fish and that they would have altered behavioural patterns (such as increased activity levels due to heightened anxiety), even in the absence of the hormonal stress response.

Methods and materials

Fish housing, husbandry and experimental setup

Wild three-spined sticklebacks were captured in the River Endrick near Killearn, Scotland, in January 2017. In total 200 fish were transported to the aquarium facilities in the Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, where they were housed in a single fibreglass holding tank with a diameter of 90 cm and a total volume of 325 L. The tank contained rocks and plastic pipes for shelter, and was constantly aerated and supplied with filtered, de-chlorinated and UV-sterilised water at 12 °C. Fish were fed *ad libitum* with frozen bloodworm. On 10th and 11th April 2017, 144 unsexed fish were randomly divided equally between the Experimental (stress-exposed) and Control (non-stress-exposed) group. Three randomly-selected fish that clearly differed in size (to allow for individual identification) were placed in each of the 24 Experimental and 24 Control clear 10 L plastic tanks (17x19x32 cm). Each tank was fitted with an air stone for aeration and a plastic plant for sheltering. The plants were removable in Experimental tanks and fixed to the bottom in Control tanks, but were otherwise identical. Experimental tanks were placed on a rack that was physically separated from the rack containing Control tanks. In addition, they were shielded with opaque black plastic to prevent the stressors from affecting the Control fish. However, all tanks were in the same room and so experienced the same ambient temperature (12 °C), background noise and photoperiod (14L : 10D, created by artificial lights). To prevent aggression and reduce social stress, any fish that developed male secondary sexual characteristics (blue colouration of eyes and/or red colouration of throat (Wootton, 1976)) during the experimental period were removed from the tanks so that the tanks only contained either female or non-reproductive male fish. Fish were fed twice daily, *ad libitum*, with frozen bloodworm and live *Artemia* nauplii.

Unpredictable chronic stress protocol

Following 14 days of acclimation to the tank and feeding regime, fish from the Experimental group were subjected to unpredictable chronic stress protocol (UCSP), loosely based on those used by Tsalafouta *et al*. (2015) and Madaro *et al*. (2015). To maintain the unpredictability and minimise the potential for habituation, the online Research Randomiser tool (Urbaniak, 2015) was used to

construct the UCSP schedule prior to the start of the experiment. The UCSP consisted of the following stressors, three of which were used each day: a) turning lights on for 30 minutes during the dark period (night), b) turning lights off for 30 minutes during the light period (day), c) increase in light intensity from 480 lux to 1320 lux for 30 minutes during the light period, d) turning lights off during the light period and flashing lights in the darkness for 10 minutes, e) increase in tank aeration to create physical disturbance for 2 x 10 seconds, with a brief period of rest in between, f) removal of shelter (artificial plant) for 15 minutes, g) chasing with a net for 2 x 30 seconds, with a brief period of rest in between, h) air exposure for 2 x 10 seconds, with a brief period of rest in between. With the exception of a) that was applied at night only, all other stressors were applied either in the morning (8:30 – 11:00), at noon (11:00 – 14:00) or in the afternoon (14:00 – 17:30). The order and timing of stressor presentation was randomised, with the proviso that only one stressor was applied at any one time point (Figure 2.1b). The UCSP continued throughout the breeding season (Figure 2.1a; see also Chapter 3) and ended on day 67 of the experiment, i.e. when the females ceased to reproduce.

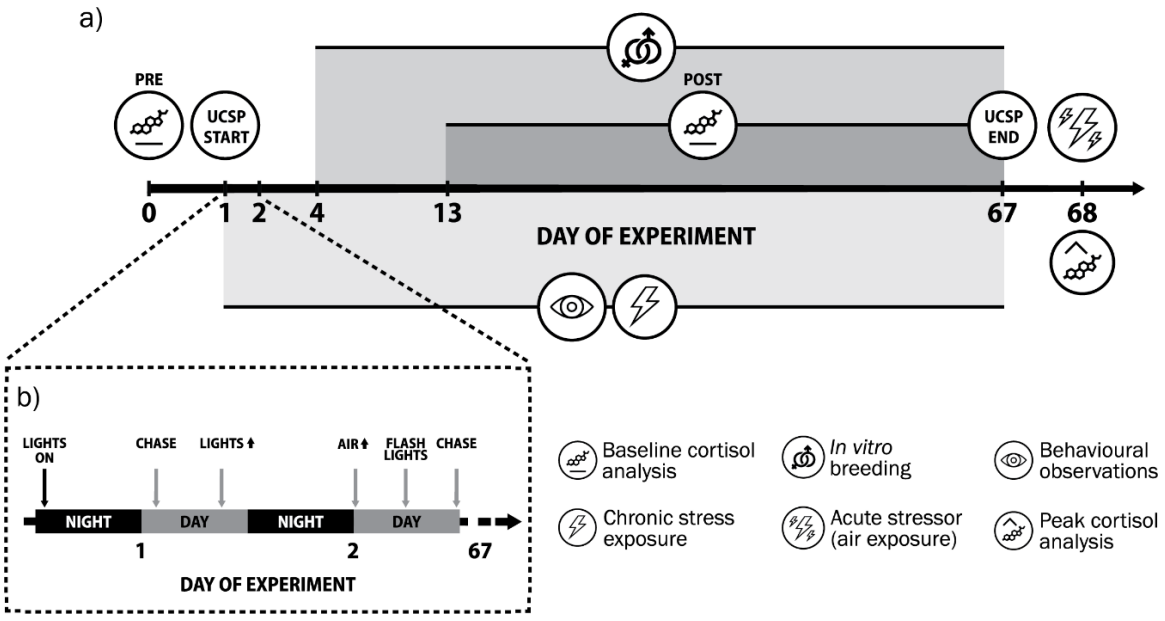

Figure 2.1 a) Experimental timeline of the unpredictable chronic stress protocol (UCSP), behavioural observations and breeding; PRE – sample taken prior to the start of the UCSP, POST – sample taken during the UCSP. Chronic stress exposure was limited to the Experimental group, both Experimental and Control fish were challenged with an acute stressor at the end of experiment. b) An example of a daily stressor schedule for the Experimental group.

Measuring primary stress response

The method of water sample collection and extraction of water-borne cortisol used in this study for measuring the primary stress response is based on Sebire *et al*. (2007, 2009), with minor refinements resulting from pilot assays. Prior to the start of the UCSP, a water sample was collected from each Control and Experimental fish to determine pre-treatment baseline cortisol levels. To obtain the samples non-invasively, 100 mL of filtered and UV-sterilised water from the same aquarium system in which the fish were held was transferred to 600 mL borosilicate beakers. Fish were netted and placed individually in the beakers for 30 minutes. In order to minimise stress on the fish, care was taken to minimise the handling time and the beakers were suspended in the original tanks (so that test fish could see their normal 'home' environment, including their tank companions). After 30 minutes, fish were released back into their tanks and each beaker water sample transferred to two 50 mL centrifuge tubes. Water samples were immediately frozen and stored at -20 °C until further analysis. A sample of water from the aquarium system (background sample) was collected at the same time to account for potential background cortisol levels.

The fish then reproduced through *in vitro* fertilisation, with each producing up to three clutches throughout the experimental period (see Chapter 3). A second water sample was collected at the time when the second clutch was produced (to standardise for female reproductive state), to measure post-treatment baseline cortisol levels. The timing of collection differed between the females, with a minimum of 11 days between the start of the UCSP and the collection of the post-treatment baseline water sample. The collection method was the same as for the pre-treatment baseline sample. Post-treatment water samples were collected at the end of the UCSP (i.e. on day 67) in the same way from all remaining fish that did not produce at least two clutches. The sex of non-reproductive fish was determined through dissection and the samples from male fish were discarded leaving samples from females that did not reproduce (n=15), or produced only one clutch during the breeding season (n=7). A third water sample was collected from a subset of the breeding females (n=27) in order to assess the effect of the UCSP on primary stress responses to an acute stressor. After the completion of UCSP, i.e. on day 68 of the experiment, each female that produced three clutches during the breeding season was subjected to an acute stressor: netting and air exposure for 2 x 2 minutes, which is known to

elicit an acute stress response in fish (Ramsay *et al.*, 2009 and a pilot study on sticklebacks). Since peak cortisol release into water has been previously shown to occur between 60 and 90 minutes after exposure of sticklebacks to stressful stimulus (Sebire *et al.*, 2007), the fish were released into their home tank for 30 minutes, prior to being placed individually in a 600 mL beaker filled with 100 mL of filtered and UV-sterilised water from the aquarium system for 60 minutes. All post-experimental baseline and acute water samples were stored at -20 °C until further analysis.

Each pre-treatment baseline, post-treatment baseline, and acute water sample, along with their respective background samples, was processed through an individual solid-phase extraction cartridge (Sep-Pak C18 Plus Light, Waters) using a 20-port vacuum manifold (Biotage VacMaster, Figure 2.2). In addition, a water sample with known cortisol concentration (1.25 µL per mL) was prepared and processed using the same method to assess extraction efficiency. Prior to the extraction, each cartridge was primed with 1 mL of 100% methanol and 1 mL of distilled water. Water samples at room temperature were processed through the cartridges at approximately 2 mL per minute, as recommended by the manufacturer for maximum retention of free cortisol fraction from the sample. Following extraction, cartridges were washed with 1 mL of distilled water and 1 mL of weak solvent (20% methanol) to remove impurities. Immediately after washing, cortisol was extracted from the cartridges with 1 mL of 100% methanol into 12x75 mm borosilicate tubes.

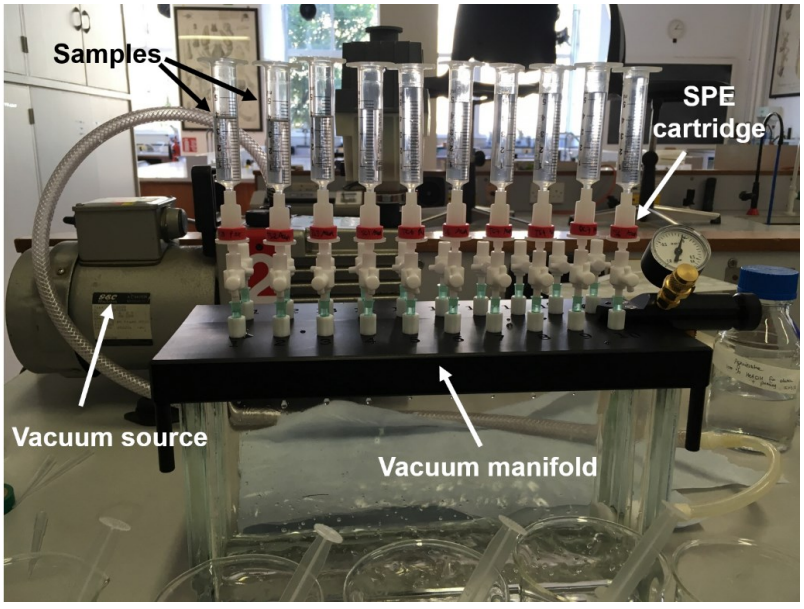

Figure 2.2 Solid-phase extraction (SPE) using 20-port vacuum manifold connected to a vacuum source.

Extracted samples were placed on a heat block at 45 °C, evaporated until dry under nitrogen gas in a sample concentrator (Techne Sample Concentrator with DB-3 Dri-Block) and reconstituted in 250 µL of assay buffer. A colorimetric competitive ELISA assay (Enzo Life Sciences, ADI-900-071) was used to quantify cortisol concentration in undiluted, reconstituted samples, which were run in duplicate on a 96-well plate along a set of cortisol standards ranging from 156 to 10,000 pg/mL. Optical density (OD) of each sample was determined using a spectrophotometer (Multiskan Spectrum, Thermo Scientific). Samples that had cortisol concentrations too high to be within the standard curve range were later diluted with assay buffer 1:4 and re-assayed. The duplicates were used to calculate the intra-assay coefficient of variation (CV). A reference sample, containing pooled cortisol extracts collected from six random stock fish, was run on each plate to determine inter-assay CV. Mean values of intra-assay CV and inter-assay CV across six plates were 8.22% and 15.14% respectively. Mean extraction efficiency was 118%.

Fish measurements and organ sampling

Following the collection of the pre-treatment cortisol sample, each female was blotted dry and its wet mass was recorded to determine its initial body mass (to 0.001g) prior to the commencement of the experimental treatments. The initial standard length (excluding caudal fin) of each female was measured (to 0.1mm) using vernier callipers. After the completion of the UCSP and breeding, all surviving females (n=57) were re-measured to determine their final mass and length. Fish were then culled by an overdose of anaesthetic (50mg/L benzocaine solution) followed by cervical dislocation. Liver and ovaries were dissected out and the wet mass of each organ was recorded. Where eggs were present in the ovaries, these were removed prior to weighing.

Behavioural observations

Observations were performed throughout the period of the UCSP to assess the impact of chronic stress on stickleback behaviour. Fish were observed in their home tanks and their movement and sheltering behaviour was scored in a series of 10 successive observations, with approx. 1 minute between the observations. A scan-sampling approach was used, and a score of “1” was assigned if the fish was changing position at the time of observation, either by propelling itself forwards or backwards or by moving up and down in the water column. If the

fish was immobile, it was assigned a score of “0”. In addition, a score of “1” was assigned if at the time of observation the fish was no more than one body length away from the shelter, i.e. the artificial plant or the outflow pipe; otherwise, a score of “0” was assigned. Each series of observations, for each tank, was repeated at least three times throughout the experimental period, with an average of 13 days between the series. Two types of behavioural observations were performed in fish from the Experimental group: at least 3 series of “stress-on” observations (during increased light intensity and shelter removal stressors, and immediately after flashing lights, increased aeration, chasing and air exposure stressors), and at least 3 series of “stress-off” observations (at least 2 hours after the previous stressor). The aim of the “stress-off” observations was to assess if potential behavioural effects of stress persist even when the intermittent and chronic stressor is not currently applied. A movement score and a sheltering score for each fish (ranging from 0 to 10), was calculated by adding the score from all 10 observations of a fish in a series. A mean score per tank (0-10) was then calculated for each series of observations by averaging the mean scores of all the fish in the tank (1-3 fish). Immediately after each series of observations of movement and sheltering behaviour, novel food (frozen daphnia) was added to the tank, the time (in seconds) taken for each fish in the tank (a maximum of three fish) to attack and swallow the food was recorded and the mean value of latency to feed was calculated for each tank. If a fish did not attack and swallow food within three minutes, the latency to feed of that fish was recorded as 180 seconds (Oswald *et al.*, 2013). The following were recorded for each series of observations: tank, Julian date of observation, time elapsed from last feeding, water temperature.

Data analysis

The MyAssays online data analysis tool (MyAssays Ltd.) was used to calculate cortisol concentrations from optical densities, using the Four Parameter Logistic Curve as per the kit manufacturer’s instructions. Samples that fell outside of the standard curve range (n=3, all pre-experimental baseline) were excluded from further analysis. Statistical analyses for cortisol were restricted to females for which all three water samples (pre-treatment baseline, post-treatment baseline and acute) were collected, and accounting for the three excluded samples this yielded a final sample size of 14 Control and 10 Experimental females. The cortisol concentration of the relevant background sample was

subtracted from each sample; an average concentration of cortisol detected in the water from the aquarium system was 0.189 ng/mL.

Linear Mixed Models (LMM) were used to analyse whether cortisol levels changed between the beginning and the end of the UCSP, and between the baseline and acute cortisol levels following the UCSP. The fixed factors included in the two initial models were treatment group (categorical variable), sampling point (pre- and post-experimental baselines, or post-experimental baseline and acute sample respectively; categorical variable), mass (covariate), and interaction terms treatment group*sampling point and treatment group*mass.

The formula CF = mass/length^a was used to determine pre- and post-experimental condition factor (CF), where the exponent *a* was the slope of the linear regression between log body mass and log body length (3.16). LMM was used to analyse the difference in CF between Control and Experimental females before and after the period of UCSP. The fixed factors included in the model for initial CF was treatment group (categorical variable) only. The fixed factors included in the model for final CF were treatment group (categorical variable), number of clutches produced by the female during the breeding season (covariate) and the interaction term treatment group*number of clutches.

The formulas GSI = 100*(gonad mass/(body mass-gonad mass)) and HSI = 100*(liver mass/(body mass-liver mass)) were used to calculate gonado-somatic index (GSI; a predictor of reproductive condition) and hepato-somatic index (HSI; a predictor of energetic status) respectively. LMM were used to analyse whether the treatments affected the GSI and HSI of the fish following the period of the UCSP. The fixed factors included in the initial models were treatment group (categorical variable), number of clutches produced by a female during the breeding season (covariate), pre-experimental body mass (covariate) and the interaction term treatment group*number of clutches.

Mean behavioural scores (movement, sheltering and latency to feed) were calculated by averaging the scores of all fish present in the tank (1-3 fish per tank), since their behaviour was not independent of one another. These averaged scores were then z-score standardised using the “sapply” function in R Base Package (R Core R, 2017) to account for differences in standard deviation. Initially, Principle Components Analysis (PC) was performed on all three behavioural scores, but due to a poor correlation between movement and

latency to feed (Pearson product-moment correlation of 0.029), the latter score was analysed separately. Sheltering score and movement score were summarised with PCA, and the resulting single principal component (PC1) was used as an index of activity in further analyses. A LMM was used to analyse the difference in activity (PC1) between Control females, stress-on observations of Experimental females and stress-off observations of Experimental females. The following fixed factors were included in the initial model: treatment group (categorical variable), Julian date of observations (covariate), temperature (covariate) and time from last feeding (covariate), as well as interaction terms treatment group*Julian date, treatment group*temperature and treatment group*time from last feeding. Tank ID was included as a random factor. Non-significant terms were removed by backwards selection. Latency to feed was analysed using LMM with the same fixed and random factors as in the activity model.

All statistical analyses were performed in R statistical software (version 3.4.3, R Development Core Team). All LMMs were fitted using the "lme4" package in R (Bates *et al.*, 2015). The degrees of freedom were estimated by Satterthwaite approximation. P values were obtained from t-statistics using the "lmerTest" package in R, with alpha level of 0.05 (Kuznetsova *et al.*, 2017). In all models Tank ID was included as a random factor to control for the potential lack of independence between the fish in a tank; Fish ID was included as a second random factor in analyses that used multiple measurements from the same female. For all models, non-significant terms were removed by backwards selection.

Results

Water-borne cortisol levels

Cortisol levels significantly increased in adult female sticklebacks between the beginning (pre-experimental baseline) and the end (post-experimental baseline) of the experimental period (Fig. 2.3). However, this increase was small compared to the substantial and significant increase in cortisol levels following acute stress exposure (comparison of post-treatment baseline and acute measurements; Table 2.1). Neither baseline nor acute cortisol levels were affected by the treatment, the body mass of the female and the interaction between these two factors (see Table 2.1).

Condition factor

Preliminary analysis revealed the presence of an abnormally high value of condition factor before the UCSP for one of the fish (more than 3 standard deviations from the mean), which affected the distribution of model residuals. This outlier was removed and data re-analysed; the original analysis is available in the Appendix 2 and found qualitatively similar results. Prior to the start of the UCSP, the condition of females from the Experimental group did not differ from those of the Control group. After a period of chronic stress exposure, Experimental females had a tendency to be in a poorer body condition than Controls, but this difference was not significant (Figure 2.4). Contrary to expectations, the number of clutches produced over the course of the breeding season (range 1-3) did not have an effect on the final CF (Table 2.2).

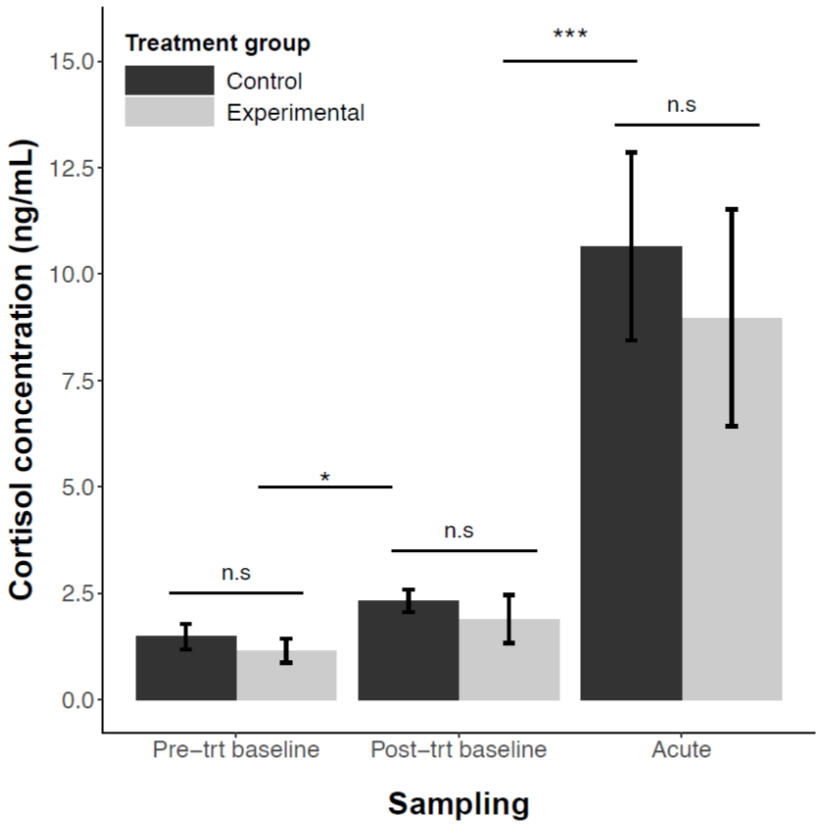

Figure 2.3 Differences in cortisol levels (expressed as ng/mL) of breeding female three-spined sticklebacks at three sampling points: pre-treatment baseline (Pre-trt, prior to breeding), post-treatment baseline (Post-trt, approx. in the middle of the breeding season) and following the exposure to an acute stressor (at the end of the breeding season), in Control (n=14; blue) and Experimental (n=10; orange) fish. Bars indicate means ± s.e.; * $p<0.05$, *** $p<0.001$; n.s. indicates non-significant result.

Table 2.1 Summary of Linear Mixed Models (LMM) used to analyse the differences in cortisol levels between different sampling points. Values give estimate ± s.e. The reference Treatment was the Control group. Female ID and tank ID were included in the models as random factors. Control group n=14 females, Experimental group n=10. Treatment group and any significant terms (indicated with p-values in underlined and bold font) were retained in the final model.

Model terms	**Difference between pre- and post-experimental baseline cortisol**	**Difference between post-experimental baseline and acute cortisol**
Treatment group	-0.402 ± 0.375, $t_{1,17.84}$=-1.072, p=0.298	-0.273 ± 0.254, $t_{1,17.92}$=-1.074, p=0.297
Sampling point	0.798 ± 0.333, $t_{1,29.71}$=2.394, **p=0.023**	1.328 ± 0.226, $t_{1,23}$=5.875, **p<0.001**
Mass	0.058 ± 0.430, $t_{1,31.13}$=0.136, p=0.893	0.271 ± 0.347, $t_{1,32.84}$=0.780, p=0.441
Treatment group*Sampling point	-0.089 ± 0.694, $t_{1,28.61}$=-0.128, p=0.899	0.181 ± 0.464, $t_{1,28.31}$=0.390, p=0.699
Treatment group*Mass	-0.009 ± 1.004, $t_{1,36.27}$=-0.009, p=0.993	0.112 ± 0.769, $t_{1,19.36}$=0.146, p=0.885

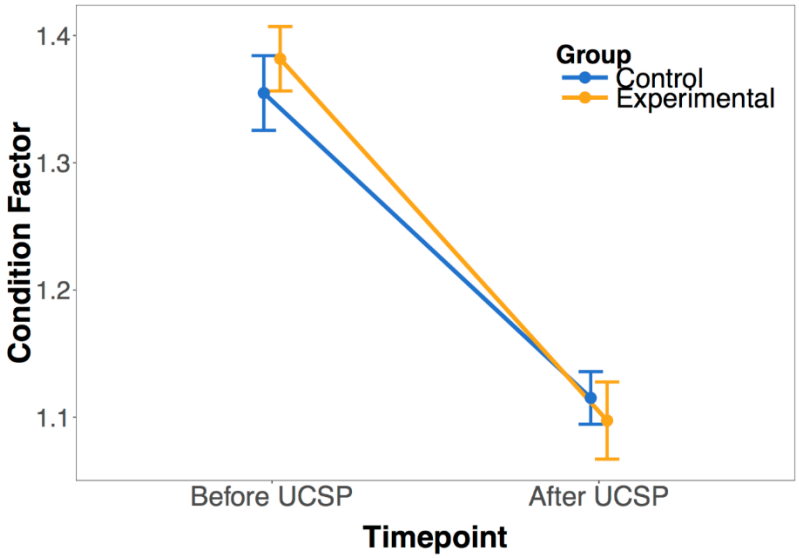

Figure 2.4 Condition factor (CF) of female three-spined sticklebacks before and after the Unpredictable Chronic Stress Protocol (UCSP) in Control (n=27; blue) and Experimental (n=29; orange) fish. The error bars represent Standard Error of the Mean (s.e.).

Table 2.2 Summary of Linear Mixed Models (LMM) used to analyse the effect of Treatment group and the number of clutches produced over the breeding season on female body condition, expressed as Condition Factor (CF). Values give estimate ± s.e. The reference Treatment was the Control group. Female ID was included in the models as a random factor. Control group n=27 females, Experimental group n=29. Number of clutches was used as a factor in the model for final (after UCSP) but not initial (before UCSP) CF. Treatment group and any significant terms (indicated with p-values in underlined and bold font) were retained in the final model.

Model terms	CF before UCSP	CF after UCSP
Treatment group	0.027 ± 0.068, $t_{1,34.28}$=0.395, p=0.695	-0.043 ± 0.026, $t_{1,55.00}$=-1.644, p=0.106
No. of clutches	n/a	-0.005 ± 0.011, $t_{1,54.00}$=-0.512, p=0.611
Treatment group*No. of clutches	n/a	-0.001 ± 0.021, $t_{1,52.94}$=-0.071, p=0.944

Gonado-somatic and hepato-somatic indices

Exposure of females to a period of chronic stress affected neither their reproductive condition (indicated by GSI) nor their energetic state (indicated by HSI; Figure 2.5, Table 2.3). GSI values were highest in females that produced three clutches during the breeding season, and females that produced at least two clutches had significantly higher values of HSI, regardless of which treatment group they originated from (Figure 2.6).

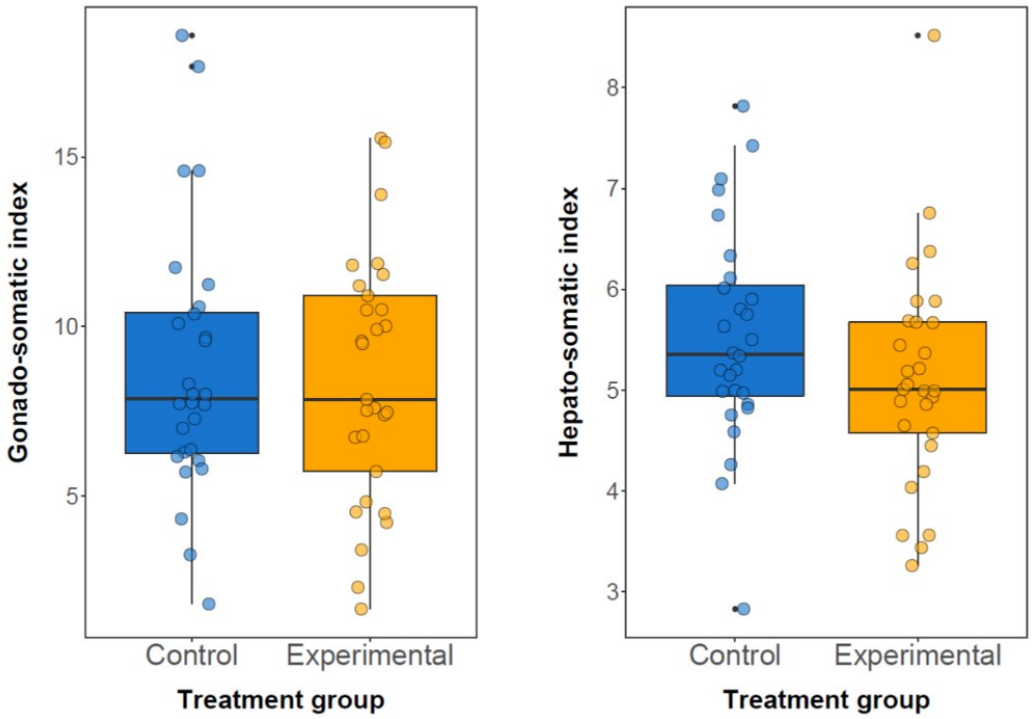

Figure 2.5 Comparison of gonado-somatic index (GSI) and hepato-somatic index (HSI) at the end of the breeding season in female three-spined sticklebacks from Control (n=28; blue) and Experimental (n=29; orange) group. Black dots represent outliers.

Table 2.3 Summary of Linear Mixed Models (LMM) used to analyse the effect of Treatment group and the number of clutches produced over the breeding season on gonado-somatic Index (as log GSI) and hepato-somatic index (HSI, untransformed). Values give estimate ± s.e. The reference Treatment was the Control group. Control group n=28 females, Experimental group n=29. Treatment group and any significant terms (indicated with p-values in underlined and bold font) were retained in the final model.

Model terms	**Gonado-somatic index (GSI)**	**Hepato-somatic index (HSI)**
Treatment group	-0.001 ± 0.143, $t_{1,27.35}$=-0.006, p=0.995	-0.315 ± 0.321, $t_{1,35.36}$=-0.982, p=0.333
No. of clutches	0.134 ± 0.511, $t_{1,52.90}$=2.614, **p=0.012**	0.231 ± 0.102, $t_{1,49.71}$=2.259, **p=0.028**
Treatment group*No. of clutches	-0.013 ± 0.104, $t_{1,51.73}$=-0.130, p=0.897	0.085 ± 0.207, $t_{1,48.37}$=0.413, p=0.682

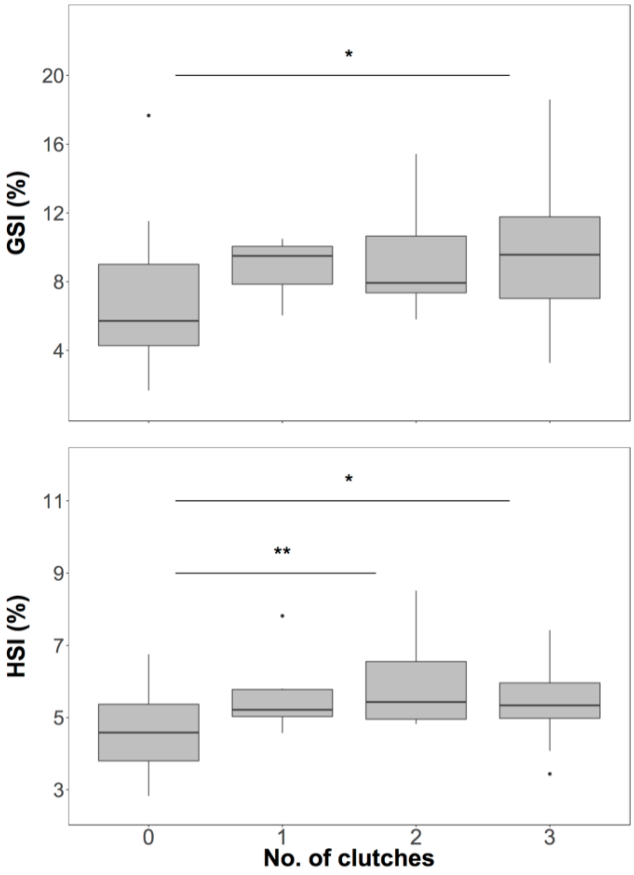

Figure 2.6 The relationship between the number of clutches produced by female three-spined sticklebacks during the breeding season and their mean gonado-somatic and hepato-somatic index. For clarity, and due to the lack of difference in GSI and HSI between the treatment groups, the figure shows combined data for the Control and Experimental fish (n=57). The significance of the effects was tested using linear regression. Non-significant relationships were omitted from the plot; significance codes: * 0.05, ** 0.01

Activity levels and latency to feed

Tank-average scores for sheltering and for movement were significantly correlated (Pearson's product-moment correlation coefficient =-0.535, df =244, $p<0.001$), justifying their inclusion in a PCA to produce a single composite variable indicating overall activity. The resulting PC1 had an Eigenvalue of 1.24 (and thus satisfied the Kaiser-Guttmann criterion of Eigenvalue > 1) and explained 77% of the total variance in the data (loading for movement = 0.707, for sheltering = -0.707). Higher PC1 scores indicate higher overall activity levels, with fish being more likely to be in motion and away from the shelter when observed. Bartlett's test of sphericity ($p<0.001$) and the Kaiser-Meyer-Olkin (KMO) test of sampling adequacy (0.5) indicated that a minimum standard was met to proceed with the results of the PCA.

However, the results should be treated with caution since KMO results between 0.5 and 0.6 are considered poor but acceptable (Kaiser and Rice, 1974). Fish

from the Experimental group showed a higher activity than Control fish while the stressors were applied (during stress-on observations; Figure 2.7, Table 2.4), but this effect did not persist between the stressors (during stress-off observations). Julian date of observations significantly affected the activity levels, with less activity later in the experimental period (i.e. later in the breeding season; Figure 2.7, Table 2.4). Time elapsed from the last feeding had a positive influence on activity (Table 2.4).

Latency to feed during stress-on observations (Table 2.5) did not differ from that observed in Control fish. During stress-off observations, fish from the Experimental group took significantly longer to attack and swallow food (Table 2.5), than did Control fish. The Julian date of observation influenced the latency score during stress-off observations, with less time required to attack and swallow food later in the experimental period (Figure 2.8). Latency to feed in Control fish and the stress-on observations of Experimental fish did not change significantly over time.

Table 2.4 Summary of Linear Mixed Models (LMM) used to analyse the variation in stickleback activity levels. The activity level (PC1) was obtained from a PCA of sheltering score and movement score. Values give estimate ± s.e. The reference observation was Control. Control: n=107 observations on 23 tanks, Experimental: n=93 observations during stress-off periods and n=46 during stress-on periods on 24 tanks. Observation and any significant terms (indicated with p-values in underlined and bold font) were retained in the final model.

Model terms	**Effect on activity levels (PC1)**
Observation: Exp. stress-off	0.285 ± 0.211, $t_{2,50.32}$=1.351, p=0.183
Observation: Exp. stress-on	0.798, ± 0.244, $t_{2,86.51}$=3.272, **p=0.001**
Julian date	-0.020 ± 0.004, $t_{1,208.86}$=-5.016, **p<0.001**
Temperature	0.381 ± 0.198, $t_{1,228.09}$=1.924, p=0.056
Time from last feeding	0.022 ± 0.008, $t_{1,224.45}$=2.773, **p=0.006**
Observation: Exp. stress-off*Julian date	-0.008 ± 0.009, $t_{2,204.65}$=-0.976, p=0.330
Observation: Exp. stress-on*Julian date	-0.020 ± 0.017, $t_{2,222.10}$=-1.197, p=0.232
Observation: Exp. stress-off*Temperature	0.016 ± 0.468, $t_{2,225.36}$=0.033, p=0.973
Observation: Exp. stress-on*Temperature	-0.880 ± 0.460, $t_{2,214.75}$=-1.912, p=0.057
Observation: Exp. stress-off*time from last feeding	-0.004 ± 0.016, $t_{2,214.17}$=-0.224, p=0.823
Observation: Exp. stress-on*time from last feeding	-0.002 ± 0.030, $t_{2,215.44}$=-0.076, p=0.939

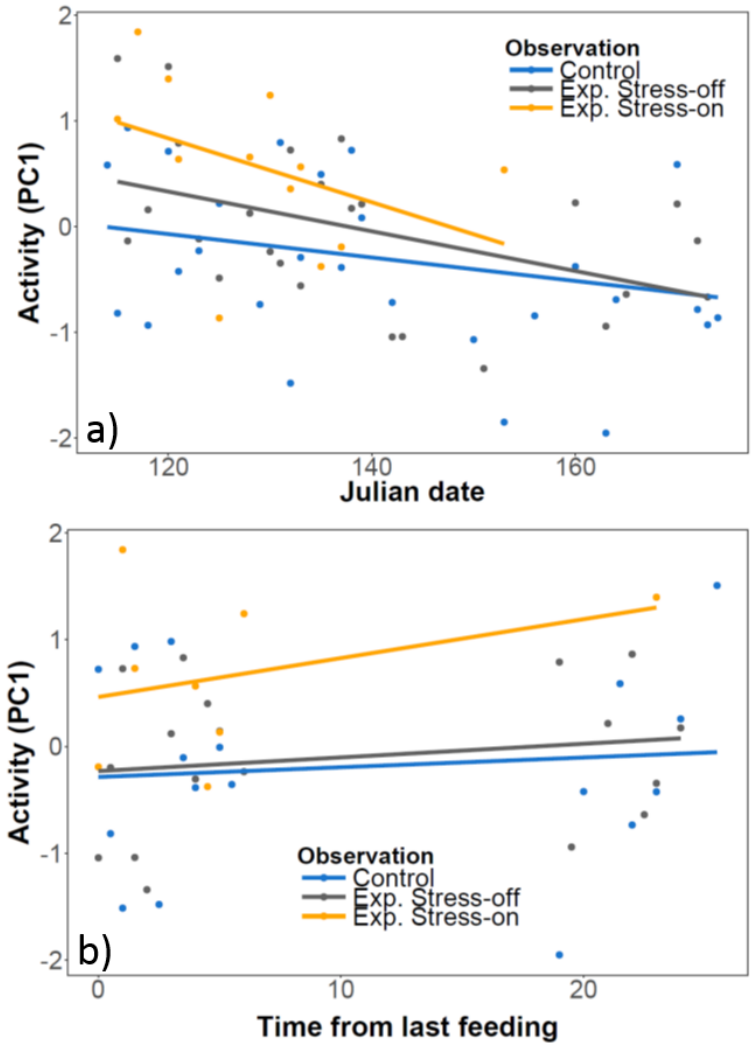

Figure 2.7 The effect of Julian date of observation (2.7a) and time elapsed from the last feeding (in hours; 2.7b) on female stickleback activity levels (PC1) across three types of observations: Control group observations (blue), Experimental group observations during stressor (Exp. Stress-on; orange) and Experimental group observations between stressors (Exp. Stress-off; grey). The activity level (PC1) was obtained from a PCA of sheltering score (square root-transformed) and movement score. For clarity, each point on the plot represents a mean value of activity at a given Julian date/time from last feeding for each of the observation types.

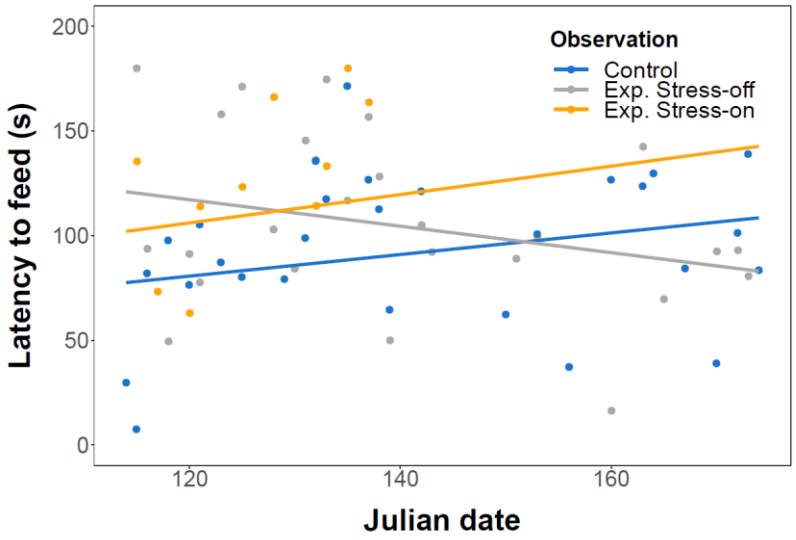

Figure 2.8 The effect of Julian date of observation on female stickleback latency to feed (expressed as the time in seconds taken to attack and swallow food) across three types of observations: Control group observations (blue), Experimental group observations during stressor (Exp. Stress-on; orange) and Experimental group observations between stressors (Exp. Stress-off; grey). For clarity, each point on the plot represents a mean value of activity at a given Julian date for each of the observation types. The regression lines were predicted from a model including observation, Julian date (and their interaction) and time from last feeding as fixed effects.

Table 2.5 Summary of Linear Mixed Models (LMM) used to analyse the variation in female latency to feed (as latency in seconds). Values give estimate ± s.e. The reference observation was Control. Control: n=107 observations on 23 tanks, Experimental: n=93 observations during stress-off periods and n=46 during stress-on periods, on 24 tanks. Observation and any significant terms (indicated with p-values in underlined and bold font) were retained in the final model.

Model terms	**Effect on latency to feed**
Observation: Exp. stress-off	148.291 ± 65.559, $t_{2,220.40}$=2.262, **p=0.025**
Observation: Exp. stress-on	-10.052 ± 121.354, $t_{2,228.33}$=-0.083, p=0.934
Julian date	0.532 ± 0.305, $t_{1,212.02}$=1.744, p=0.083
Temperature	5.841 ± 10.947, $t_{1,229.01}$=0.534, p=0.594
Time from last feeding	-0.779, ± 0.438, $t_{1,226.88}$=-1.777, p=0.077
Observation: Exp. stress-off*Julian date	-0.955 ± 0.468, $t_{2,215.67}$=-2.042, **p=0.042**
Observation: Exp. stress-on*Julian date	0.276 ± 0.930, $t_{2,227.12}$=0.296, p=0.767
Observation: Exp. stress-off*Temperature	-16.435 ± 26.904, $t_{2,220.49}$=-0.611, p=0.542
Observation: Exp. stress-on*Temperature	28.581 ± 25.773, $t_{2,211.09}$=1.109, p=0.269
Observation: Exp. stress-off*time from last feeding	1.423 ± 0.914, $t_{2,217.85}$=1.556, p=0.121
Observation: Exp. stress-on*time from last feeding	-0.400 ± 1.660, $t_{2,221.45}$=-0.241, p=0.810

Discussion

Baseline and acute cortisol levels

The results of this study clearly show an increase in baseline cortisol levels in adult female sticklebacks during the course of the breeding season. However, this increase was the same independent of treatment group, and therefore it was unlikely to be caused by exposure to chronic environmental stressors. Instead, I propose that the observed cortisol elevation in both treatment groups may be a result of the reproductive process itself. It has been previously shown that sexual maturation and reproduction are correlated with elevated glucocorticoid levels (Baker *et al.*, 2013, Jamalzadeh *et al.*, 2013, Lattin *et al.*, 2016, Casagrande *et al.*, 2018). Generally, higher glucocorticoid levels in vertebrates are negatively correlated with reproductive function, leading to reproductive suppression and reduced fitness in stressed individuals (Ricklefs and Wikelski, 2002, Wingfield and Sapolsky, 2003, Bonier *et al.*, 2009). However, despite being statistically significant, the increase in cortisol levels between pre-breeding and breeding periods was small in biological terms (an average increase of 0.79 ± 0.35 ng/mL, or 40% of initial values) and therefore unlikely to affect female reproductive ability.

Overall, there is currently a lack of consensus as to the direction and magnitude of vertebrate hormonal response to chronic stress, with animals showing either increase, decrease or no change in baseline glucocorticoid levels, depending on species, context, method used to induce chronic stress etc. (Dickens and Romero, 2013). For example, my results contrast with those of Tsalafouta *et al.* (2015), who found a significant increase in baseline cortisol release by sea bass following exposure to chronic stress. Similarly, a study on Atlantic salmon has shown that challenging fish with an unpredictable chronic stress protocol led to elevation of cortisol (Madaro *et al.*, 2015). In both studies, fish were exposed to chronic stress at early life stages only (larvae and parr respectively), unlike sticklebacks in my study, which experienced stress as sexually mature individuals. This may explain the disparities between these studies, as life stage is a major factor explaining differences in fish response to stressors (e.g. Koakoski *et al.*, 2012). In addition, stress indices and endocrine response to stressors may differ between wild and captive animals, as demonstrated in various vertebrate taxa (Woodward and Strange, 1987, Congleton *et al.*, 2000,

Coburn *et al.*, 2010, Fallahsharoudi *et al.*, 2015, Ericsson and Jensen, 2016). Animals in the wild are often subjected, and thus accustomed, to unpredictable and fluctuating environmental conditions, which could explain the lack of physiological response of wild-caught sticklebacks to the UCSP.

The apparent lack of effect of chronic stress exposure on cortisol levels in the Experimental group may also be a consequence of the nature of the experimental design. In the current study baseline cortisol level was measured at only one time point during the period of stress exposure, i.e. at the time of second spawning. In vertebrates, chronic stress exposure may result in alteration of the stress axis function (Rich and Romero, 2005), including exhaustion of the HPA/HPI axis and fall in plasma cortisol levels over time (Madaro *et al.*, 2015). An analogous situation may have occurred in my study; this however does not explain the subsequent increase in cortisol level after challenging fish with an acute stressor, suggesting that the attenuating effect of chronic stress on the HPI axis was temporary or that the fish habituated to the stressors on a physiological level. Measuring cortisol at regular intervals from the beginning of the UCSP would potentially provide a more complete picture on baseline glucocorticoid levels in response to chronic stress.

Challenging female sticklebacks to an acute stressor led to a significant elevation of water-borne cortisol levels, independent of the treatment group. This result differs from previous studies on fish (Jeffrey *et al.*, 2014, Madaro *et al.*, 2015) and other vertebrates (Rich and Romero, 2005, Crespi and Warne, 2013), in which animals exposed to chronic stress had diminished response to a subsequent acute stressor. Such a reaction is expected when prolonged stimulation leads to exhaustion of the HPI axis (Romero, 2004). In the present study, the response of stress-exposed fish did not exceed that of non-exposed ones, but neither was it excessively blunted. One possible explanation may be partial habituation of the sticklebacks to chronic stress, which relieved the pressure on the HPI axis. This would be evident if the fish showed a variation in acute stress response at various time points during the experiment; however, in my study the acute response was measured at only one time point at the end of the UCSP. It is therefore impossible to determine whether it changed over the course of the experiment, and this question should be addressed in future studies. It is also likely that the response to subsequent acute stressor is highly dependent on the duration, nature and intensity of the preceding chronic stress

and therefore direct comparison of studies using different chronic stress protocols may not be feasible.

An additional factor that should be taken into account when measuring hormonal response of fish living in groups is cortisol coregulation, where animals living in pairs or groups up- or down-regulate each other's cortisol levels. It has been extensively studied in humans and non-human primates (DeVries *et al.*, 2003, Neu *et al.*, 2009, Saxbe *et al.*, 2015), and recently described in fish (Furtbauer and Heistermann, 2016). Most of the sticklebacks used in my study were housed in groups of at least two fish, and therefore there was a potential for fish to influence the response of each other to stressful conditions during the UCSP. This would be particularly evident in post-experimental baseline cortisol levels, but could also affect the response to an acute stressor.

Relationship between chronic stress, condition factor, GSI and HSI

In this study, I used condition factor as an indicator of female physiological state following the period of chronic stress exposure. Measures of body condition are commonly used in this context as it has been shown to be correlated with glucocorticoid levels (Froese, 2006, Baker *et al.*, 2013). Prolonged exposure to stressors affects condition factor mainly via hormone-induced metabolic changes, such as an increased breakdown of glycogen in tissues (Barton, 2002). Increased energy expenditure to fuel an elevated metabolic rate results in the depletion of energy resources and poorer body condition (Lambert and Dutil, 1997, van de Pol *et al.*, 2017). In addition, fish exposed to chronic stress have been shown to alter their feeding behaviour and reduce their food intake, resulting in reduced growth and poorer condition (Leal *et al.*, 2011, Chang *et al.*, 2018). The results of my study are only partially congruent with this hypothesis, with CF of sticklebacks exposed to the UCSP having a tendency to be lower than CF of the control females. Should this difference be statistically significant, it would support the results found by Dutta *et al.* (2005) in Rohu, by Hosoya *et al.* (2007) in haddock and by Leal *et al.* (2011) in seabass. Spencer *et al.* (2008) however found no effect of chemical stress exposure on CF in sculpins. These contradictory effects indicate that care must be taken when interpreting the results, as these may be highly context-sensitive and dependent on the type of stressor or method of stress induction. In addition, the secondary stress response (e.g. metabolic changes) is not always directly linked to the tertiary

stress response (e.g. growth or condition), for example when stress-induced metabolic changes do not result in hindered growth or condition (Van Weerd and Komen, 1998). At the other end of the spectrum is the situation described by Jentoft *et al*. (2005) in perch: after the initial, stress-induced increase in glucose generation, the fish ceased to mount this secondary stress response but their growth remained negatively affected by repeated stress exposure.

In addition to the effects of chronic stress exposure on CF, I also explored the possibility that females with higher reproductive output invest less in self-maintenance and therefore are in poorer body condition at the end of the breeding season. Because energy is a finite resource, a trade-off between fecundity and growth/condition is known to occur in a wide range of fish species (Lambert *et al.*, 2003, Brosset *et al.*, 2016). This trade-off is particularly pronounced in annual fish, i.e. ones that produce offspring over a single breeding season only, with a resulting depletion of energetic resources and deterioration of the body condition at the end of the season (Poizat *et al.*, 1999). The fish used in the present study originate from a population in which the majority of females have just a single spawning season (Lee *et al.*, 2012); therefore, I expected to see a negative correlation between the number of clutches produced by females and their CF at the end of the breeding season. Contrary to these expectations, the reproductive output had no effect on the final CF, with all females showing a decline in body condition, regardless of the number of clutches they produced. However, for some of the females in my study the end of the experimental breeding season may not have been equivalent to the natural end of their reproductive attempt. It is thus possible that in these females the expected decline in CF would be only be apparent after the last spawning, should they be allowed to continue producing clutches.

Gonado-somatic index (GSI) and hepato-somatic index (HSI) are often used along with the CF to assess the physiological state of fish under various environmental conditions (Spencer *et al.*, 2008, Leal *et al.*, 2011, Gandar *et al.*, 2017). In this study, I used GSI and HSI as a proxy of reproductive condition and overall energetic state, respectively, to assess the effect of chronic stress exposure on these two physiological measures. Glucocorticoids are known to negatively affect reproduction in vertebrates, by influencing energy metabolism and diverting resources away from the reproductive system (Bonier *et al.*, 2009). However, some individuals and populations developed resistance mechanisms

that allow them to maintain high reproductive condition and fecundity (Wingfield and Sapolsky, 2003). In my study, exposure to the UCSP did not have an effect on stickleback GSI at the end of the breeding season. This is congruent with the results of Contreras-Sanchez *et al.* (1998) in rainbow trout, but in contrast with Thomas *et al.* (2008) who observed a decline in GSI following hypoxic stress in Atlantic croaker.

An elevation in circulating glucocorticoids may also lead to an alteration in energy reserves, evident in a change in HSI. Some of the effects of disturbance on energy metabolism include downregulation of hepatic glycogen content (Chang *et al.*, 2018), hepatic proteolysis (Mommsen *et al.*, 1999, Pottinger *et al.*, 2002) and histopathological changes to liver tissue (Harper and Wolf, 2009), all of which have a potential to reduce liver mass. As a result, it is reasonable to anticipate that the HSI of stress-exposed fish would be lower than in non-exposed fish. In my study, there was no significant effect of chronic stress exposure on energetic state indicated by HSI. A similar lack of effect of stress on the HSI was observed by Leal *et al.* (2011) in sea bass, but other studies provide a conflicting evidence of this effect. For example, Barton *et al.* (1987) observed a downward trend in the HSI of stress-exposed rainbow trout, whilst Gandar *et al.* (2017) reported an increase in the HSI of goldfish exposed to pollutant stress.

The current body of evidence does not provide a consensus as to the effects of stress on physiological indices, such as GSI and HSI, which vary considerably not only across contexts and species, but also on a temporal scale within species (e.g. Jamalzadeh *et al.*, 2013). In addition, other factors may confound the observed relationships and prevent clear interpretation. For example, in my study, indices of both reproductive condition (GSI) and energetic state (HSI) were correlated with the number of clutches produced by females throughout the breeding season, which may suggest that females that are in better condition have higher fecundity. However, these are merely correlations, which do not prove the causal nature of the relationships.

Behavioural effects of chronic stress exposure

Various aspects of the behavioural phenotype of vertebrates have been used as a proxy for acute or chronic stress. These include locomotor activity (Warwick *et al.*, 2013, Millot *et al.*, 2014, Krause *et al.*, 2017), cognitive abilities (Piato *et al.*, 2011, Boogert *et al.*, 2018), feeding behaviour (Leal *et al.*, 2011, Favreau-

Peigne *et al.*, 2014) and aggression (Lucion and Vogel, 1994, Øverli *et al.*, 2002), and are often combined as measures of activity or boldness (Sopinka *et al.*, 2016a, Ward-Fear *et al.*, 2018). There are many mechanisms through which exposure to stressors can affect behaviour, ranging from sensory and neurological disruption to metabolic changes (Scott and Sloman, 2004). Recent evidence from gilthead sea bream shows that early life exposure to unpredictable chronic stress conditions has far ranging neurobiological implications in the limbic area of the fish forebrain (Vindas *et al.*, 2018) and in the hippocampus area of a bird brain (Gualtieri *et al.*, 2019). One of the functions of the forebrain is the regulation of cognitive function and behaviour (Aronson, 1967, Atoji and Wild, 2006); it is therefore expected that stress-induced changes in this region will markedly influence behavioural phenotype.

Here I explored potential behavioural changes in sticklebacks following a period of chronic stress exposure. I measured their activity and feeding behaviour in two contexts: while the stressors were applied and during the resting period between stressors. As expected, fish from the Experimental group exhibited greater activity while subjected to stressors (stress-on observations); this effect however did not persist during the intervals between the appearance of the stressors (stress-off observations). There was a tendency for stress-exposed fish to have higher activity levels during stress-off observations cf. the Control fish, but it was not statistically significant. These results are somewhat consistent with the behavioural response of rainbow trout to short-and long-term exogenous cortisol treatment, where an increase in locomotor activity was observed following the short-term but not the long-term exposure (Øverli *et al.*, 2002). Conversely, a 14-day exposure to unpredictable chronic stress reduced the locomotion and exploratory behaviour of zebrafish but the same stressor had no effect when it lasted for 7 days (Piato *et al.*, 2011). Considering that stress response itself is energetically expensive (Romero, 2004, Picard *et al.*, 2018), it would be reasonable to assume that animals under direct (as in case of stress-on observations in my study) or short-term stress exposure will mount a behavioural response that is analogous to an acute stress response, e.g. increased activity. However, when faced with longer-term stressful conditions, it might be more adaptive to show normal or lower activity levels as a strategy to cope with an increased energy demand. In the present study activity levels in both Experimental and Control fish declined over time, which may indicate a strategy

to cope with the energetic demands of reproduction (Schreck, 2010, Hayward and Gillooly, 2011). Alternatively, the temporal pattern of decline in stickleback activity levels may be indicative of the onset of senescence, which is associated with reduced activity and physical ability (Carter *et al.*, 2002, Zhdanova *et al.*, 2008, Gilbert *et al.*, 2014).

The feeding behaviour of Experimental fish during the stress-on observations did not differ from that of Control fish. Despite this, chronic stress exposure had an effect on this aspect of stickleback behaviour by reducing the latency of Experimental fish to feed in the resting period between stressors later in the experimental period. Possible explanations of this change include gradual behavioural acclimation to stressful conditions or an attempt to maximise food intake and replenish energetic stores during the resting period in anticipation of future exposure to stressors, with the latter being a potential indicative of a persistent behavioural effect of chronic stress exposure. Alteration of feeding patterns in response to simulated stress has been previously reported in cortisol-implanted rainbow trout which showed a significant reduction of appetite correlated with increase in plasma cortisol (Gregory and Wood, 1999). Japanese quail exposed to unpredictable stress also showed altered dietary preference and food intake (Favreau-Peigne *et al.*, 2014).

However, the relationship between physiological stress and behavioural responses is a complex issue due to the great diversity of stress-coping strategies amongst vertebrates (Øverli *et al.*, 2007, Dingemanse *et al.*, 2010), and the possibility of habituation to stressors at a hormonal level but not at a behavioural level (Cyr and Romero, 2009); thus whilst in some animals endocrine and behavioural stress responses are correlated (Atwell *et al.*, 2012), in others these two aspects can be independent of each other (Apfelbeck *et al.*, 2011). Yet another confounding factor is intra-species variation brought about by environmental and phenotypic factors. The response to a stressful environment has been shown to be influenced by earlier experience with both stress and predation risk (Millot *et al.*, 2014, Carlson *et al.*, 2017), as well as by the early-life environment and population of origin (Bell *et al.*, 2010, Bonnot *et al.*, 2018). In three-spined sticklebacks among-individual variation in cognitive function is associated with differences in their response to changes in environmental conditions (Bensky *et al.*, 2017), and individual sticklebacks have different rates of habituation to various environmental stimuli (Bell and Peeke, 2012), which

may further confound the observed relationships. To fully account for the behavioural patterns that I detected, it would be beneficial to use fish that experienced the same rearing conditions, e.g. fish bred in constant laboratory conditions, and to consider among-individual variations in behaviour.

In summary, in this study I measured primary and tertiary response of sticklebacks to a period of unpredictable chronic stress exposure. My results suggest that baseline cortisol levels increase during the breeding season and that fish mount a significant hormonal response to an acute stressor. However, I did not detect a significant effect of chronic stress on female condition and on baseline and peak cortisol levels. This may be due to exhaustion of the HPI axis, habituation to the stress protocol or higher resilience of wild sticklebacks to unpredictable environmental conditions in terms of their physiological stress response. I did however observe an influence of chronic stress exposure on female feeding behaviour. Moreover, there was an increase in activity levels of the Experimental fish, but only during the presentation of stressors. This may indicate trade-offs between energy-demanding activities when faced with protracted stress, the response to which may be energetically expensive in itself.

Chapter 3: Effect of chronic stress on female reproductive strategies across the breeding season

Abstract

Environmental stressors have been shown to have a negative effect on fish physiology, behaviour and reproduction. Stress experienced by females during the period leading up to spawning can also alter offspring development and survival, as well as various phenotypic and behavioural aspects. However, this has usually been studied in the context of acute stress, and little is known about the effects of protracted mild maternal stress. In fish species producing multiple clutches in a single breeding season, the effects of maternal stress exposure may accumulate or diminish over time, differentially affecting the offspring from successive clutches.

I tested these effects in three-spined sticklebacks by exposing females to unpredictable chronic environmental stressors in the period leading to egg production and spawning. I measured cortisol levels in unfertilised eggs and analysed clutch phenotype in terms of clutch size and mass, single egg volume and mass, offspring development time, size at hatching, survival and growth rate.

Stress-exposed females did not deposit more cortisol in their eggs, but I observed seasonal trends in cortisol levels, unrelated to maternal treatment. Exposure of females to chronic stress altered the relationship between egg mass and clutch size, so that larger clutches of these females consisted of heavier eggs. I also observed a negative relationship between maternal mass and offspring development time and between maternal mass and fry size at hatching, independently of maternal or egg cortisol. Single egg mass and fry size at hatching increased across the sequence of clutches, but there was no correlation between these traits and egg cortisol. Female sticklebacks therefore adjust clutch and offspring phenotype over the course of a breeding season, an effect that is modified by exposure to chronic stress but likely not driven by cortisol.

Introduction

Environmental factors and evolutionary processes, working in synergy, result in an astonishing variety of life histories and reproductive strategies across the animal kingdom, not only between various taxa and species, but also within species and populations. Intraspecific variation in reproductive strategy has been demonstrated in a wide range of oviparous and viviparous animals (see reviews by e.g. Williams, 1994, Chambers and Leggett, 1996, Blanck and Lamouroux, 2007, Warne and Charnov, 2008). A large proportion of this variation can be attributed to variation in the environment experienced by parents, from large scale geographical (Kokita, 2003) and seasonal (Clarke, 1989) variations to smaller scale local effects, such as habitat quality (Woodward, 1982) and food availability (Ballinger, 1977). These environmental factors may act upon parents by eliciting hormonal responses, which lead to shifts in resource allocation, breeding behaviour and fitness (Ketterson and Nolan, 1992, Vitousek *et al.*, 2014).

One of the driving forces of the intraspecific variation in reproductive strategies is exposure to stressful environmental conditions. These include fluctuations in temperature, predation, social stressors, anthropogenic stressors, disease, etc., acting individually or synergistically on varying time scales, and leading to acute or chronic stress responses (Baker *et al.*, 2013). The primary system underlying the response to environmental stressors in vertebrates is activation of the hypothalamic-pituitary-adrenal (HPA) axis (or hypothalamic-pituitary-interrenal axis, HPI, in fish), which ultimately results in biosynthesis of glucocorticoid hormones (GCs), such as cortisol (Wendelaar Wendelaar Bonga, 1997, Sapolsky *et al.*, 2000, Romero, 2004). GCs regulate a wide range of physiological and behavioural features, including energy metabolism, immune function, growth, cognitive function and locomotor performance, etc. (Mommsen *et al.*, 1999, Barton, 2002). Combined, these factors may contribute to shifts in reproductive strategies, either across a single breeding season or across the animal's reproductive lifespan (Crespi *et al.*, 2013).

Whilst GCs confer short-time benefits for the organism by initiating a physiological response for coping with stressors, long-term elevation of GC levels tends to be negatively correlated with fitness (Bonier *et al.*, 2009, Brosset *et al.*, 2016). Many of the stress-coping strategies, which include a range of stress

hormone-mediated metabolic and cardiovascular changes (Sapolsky *et al.*, 2000, Barton, 2002), are energetically costly and may divert energetic resources away from reproductive function (Schreck *et al.*, 2001, Tilbrook *et al.*, 2002, Leatherland *et al.*, 2010). In addition, exposure to stressors often results in poorer body condition (Hosoya *et al.*, 2007, Leal *et al.*, 2011), and more localised morphological and physiological effects, such as changes in the ovarian function (Tilbrook *et al.*, 2002, Leatherland *et al.*, 2010). Therefore, a proximate cause of stress-induced alteration of an animal's reproductive strategy, such as the timing of reproduction, gamete size and quality, etc. (Schreck *et al.*, 2001) can be pinned down to the trade-off between reproduction and self-maintenance.

Many of the stress-related alterations to reproductive strategies can be categorised as non-genetic maternal effects. It is widely accepted that mothers may attempt to adjust the phenotype of their offspring to suit the prevailing or anticipated environmental conditions (Mousseau and Fox, 1998, Groothuis *et al.*, 2005, Dantzer *et al.*, 2013), although the adaptive potential of this programming may be highly context dependent (Uller *et al.*, 2013, Sheriff *et al.*, 2018). It has been previously demonstrated in egg-laying vertebrates that increased GC levels in breeding females can be reflected in the GC levels of their eggs and can have profound consequences for their offspring (Hayward and Wingfield, 2004, Love *et al.*, 2005b, Giesing *et al.*, 2011). Therefore, females can potentially adjust their reproductive strategies and developmental trajectories of their offspring through hormone-induced alteration of clutch and egg phenotype, such as clutch size, egg size or size of the offspring at birth, with the potential to program the phenotype of their offspring so as to best cope with the environment they will face. However, whether these maternal adjustments are actually adaptive or maladaptive for the offspring depends largely on a combination of maternal and environmental conditions, and the way in which the GCs are transferred into eggs. Female reproductive strategy and the phenotypic response of the offspring may be related to the kind of stress experienced by the female: the evidence from biomedical research shows that whilst acute stress has the potential to be adaptive, chronic stress tends to be much more detrimental (Romero, 2004). Adaptive potential of maternal stress is also largely dependent on mother-offspring environmental matching (Marshall and Uller, 2007, Hoyle and Ezard, 2012, Uller *et al.*, 2013); therefore, if stressors act in a long-term and

unpredictable manner, it may be reflected in heterogeneity of female reproductive strategies (Shama, 2015). In addition, there may be an indirect maladaptive effect of female stress-exposure on her reproductive strategy and the phenotype of her eggs/offspring. For example, if nutritional status and body condition of a female are negatively correlated with her levels of stress (Barton, 2002, van de Pol *et al.*, 2017), she can produce offspring that are suboptimal in terms of their number or size (Reznick *et al.*, 1996, Warner *et al.*, 2007, Wang *et al.*, 2017b), independently of maternal or egg GC levels.

In fish, as in other vertebrates, the nature of adjustments to female reproductive strategy seems to be highly species- and context-dependent, with as yet no clear consensus as to how female reproduction responds to stress and whether this response is adaptive or maladaptive. Females exposed to stressful conditions prior to spawning have a tendency to produce fewer eggs per clutch (Mileva *et al.*, 2011, McConnachie *et al.*, 2012, Mukherjee *et al.*, 2014), but this may depend on the nature and intensity of the stressors. For example, chronic stress due to subordinate social status had no effect on the clutch size in zebrafish (Jeffrey and Gilmour, 2016). Pre-spawning stress in rainbow trout was negatively correlated with egg size, but only when stressors were applied at the early stages of yolk deposition (Contreras-Sanchez *et al.*, 1998). In addition, fertilisation success of eggs from stress-exposed mothers has been shown to be reduced (Eriksen *et al.*, 2015) or not affected (Sopinka *et al.*, 2016b), depending on the context. At least some of the adjustments can be due to elevated GCs in eggs, with egg cortisol levels often being correlated with levels in the mother (Stratholt *et al.*, 1997, Eriksen *et al.*, 2006). Elevated egg cortisol levels have been shown to have no impact on the rate of early development (Stratholt *et al.*, 1997, Capelle *et al.*, 2016), and they were either positively (Capelle *et al.*, 2016) or negatively (Eriksen *et al.*, 2006) correlated with embryonic survival. In addition, elevated egg cortisol levels are associated with reduced hatching rates (Li *et al.*, 2010) but either reduced (McCormick, 1999, Sopinka *et al.*, 2016b) or increased (Mukherjee *et al.*, 2014) size at hatching. GCs in eggs can therefore have wide-ranging implications for egg phenotype and embryo development, but their effect can be difficult to disentangle from other maternal stress effects, such as reduced maternal condition (e.g. Reznick *et al.*, 1996, Weber and Brown, 2012). There is also emerging evidence of an active regulation of egg cortisol concentration by females (Faught *et al.*, 2016) and developing embryos

(Paitz *et al.*, 2016). This can explain why there may be no observable elevation of egg cortisol following maternal stress exposure (Mileva *et al.*, 2011, Cortez Cortez Ghio *et al.*, 2016).

The methods employed to simulate stressful environment may contribute to the inconsistency in results: for instance, it is unclear if cortisol from implants is released at a constant and biologically relevant rate (Crossin *et al.*, 2016), thus mimicking chronic stimulation of the HPI axis, and so these may not have the same effect on female reproduction as stressors normally encountered by fish. Similarly, artificial elevation of cortisol levels by exposing ova to exogenous cortisol removes other factors, e.g. epigenetic or nutritional, that may be important in translating maternal stress into developmental trajectories and phenotype of their gametes and offspring. This may confound the observed relationships between maternal cortisol, egg cortisol and alterations of the reproductive strategy following maternal stress exposure. Exposure of females during the breeding season to stressors, mimicking as closely as possible natural unpredictable environmental conditions, may therefore be crucial to understanding how their reproductive strategy responds to chronic stress.

Stressors may have contrasting effects on reproducing animals dependent on the time in the breeding season that stress is experienced. In particular, very little is known about the impact of stress on the reproductive strategies in species that produce a sequence of clutches across a prolonged breeding season. Certain clutch and egg characteristics, including egg size (Daoulas and Economou, 1986, Clarke, 1989) and larval size at hatching (Castillo-Hidalgo *et al.*, 2018) show strong seasonal trends, but this is usually attributed to the changes in food abundance and temperature (Bagenal, 1971, Clarke, 1989). Seasonal trends in litter size, driven primarily by maternal age at parturition, have been reported in mice (Havelka and Millar, 2004). Maternal age effects may therefore be a possible explanation of the observed patterns in fish producing clutches over a period of time. It is, however, unclear if and how these natural seasonal fluctuations are modified by female exposure to stressful conditions during the breeding season, especially when this exposure is chronic and unpredictable. McCormick (1999) reported cortisol-driven, between-clutch variation in larval size at hatching using Ambon damselfish, while Mileva *et al.* (2011) provided evidence that eggs from successive clutches produced by stress-exposed daffodil cichlids differ in size and cortisol content. However, these studies use exposure

to exogenous cortisol *in ovo* and repeated exposure to the same acute stressor, respectively. Thus, it remains unclear if relatively mild chronic stressors lead to an alteration of female reproductive strategy on a seasonal timescale.

In this study, I explore seasonal changes in female reproductive strategies of an iteroparous fish species, the three-spined stickleback. I investigate various aspects of the clutch phenotype (clutch size, egg volume, mass of single eggs) and offspring developmental trajectory (development time, hatching rate, fry size at hatching) in three successive clutches produced over a single breeding season. Furthermore, I address the largely unanswered question of how seasonal patterns in female reproductive strategy are altered by stress, by exposing females to unpredictable chronic stressors throughout the entire breeding season. The primary objective was to determine if chronic stress-exposed females deposit more cortisol in their eggs than non-stress-exposed ones, and if maternal stress affects clutch phenotype and offspring developmental trajectory. Another major objective was to examine whether any potential effects of chronic stressors show seasonal fluctuations, for example by accumulating or diminishing over time, or across the sequence of clutches produced by a female.

Methods and materials

In vitro fertilisation

This experiment used the same wild-caught three-spined sticklebacks described in Chapter 2, and details of fish husbandry and experimental set up are provided in that chapter. Following a period of acclimation to the tank conditions, equal numbers of these fish were randomly allocated to either a Control group or the Experimental treatment group subjected to unpredictable chronic stress protocol (UCSP). The UCSP lasted 67 days and was based on the protocols used by Tsalafouta *et al*. (2015) and Madaro *et al*. (2015); it is described fully in Chapter 2.

Prior to the start of the experiment, randomly selected males that expressed nuptial colouration (red throat and blue eyes (Wootton, 1976)) were removed from the non-stressed stock population, placed in individual clear 10 L plastic tanks (17x19x32 cm) and provided with nesting material: sterilised sand and green polyester thread. This pool of mature males was subsequently used to fertilise clutches produced by the Experimental and Control females; only males that built a nest were used, and the pool was continually topped up with fresh mature males from the stock population throughout the breeding season. From the first day of the UCSP, females were assessed daily for the signs of gravidity and readiness to spawn: expanded abdomen and dilated anal papilla (Wootton, 1976). The assessment was carried out visually without catching the fish in order to minimise additional causes of stress. Gravid fish were removed from their home tank for water-borne cortisol sampling (see Chapter 2) prior to stripping and fertilisation of their clutch. A single male from the pool of mature males was randomly selected to provide sperm for each clutch, and was killed by an overdose of anaesthetic (50mg/L benzocaine solution) and severing of the spinal cord. Its testes were dissected out and placed individually in Eppendorf tubes with 1mL of non-activating solution until ready to use; the solution contained salts and glucose and prevented premature activation of the sperm cells (Fauvel *et al.*, 1999, Mehlis and Bakker, 2014). Each male was used to fertilise two clutches (one testis per clutch) to reduce the number of animals used.

Females were lightly anesthetised in benzocaine solution, blotted dry and weighed (to 0.001g), and their standard length (excluding caudal fin) was measured (to 0.1mm) using Vernier callipers. The eggs were then stripped onto

the lid of a 35mm Petri dish by gently pressing the abdomen above the swelling. Stripped fish were re-weighed and the mass post-stripping was subtracted from the mass pre-stripping to estimate the mass of the clutch. The eggs were spread in a single layer on a Petri dish lid using fine brushes and photographed on a lightbox with a piece of millimetre paper for scale. Each clutch was then divided approximately in half, and a proportion of the eggs was placed in a screw-cap micro tube and immediately frozen. These eggs were stored at -80 °C for the analysis of cortisol levels. The remaining eggs were re-photographed to obtain the exact number of fertilised eggs and placed in a 35mm Petri dish containing 1 mL of tank water. A single testis was homogenised in the non-activating solution with a disposable tissue grinder and the resulting solution transferred into the Petri dish with eggs using a pipette. The dish was first agitated for 30 seconds to ensure good mixing of eggs and sperm and then left for 30 minutes at ambient temperature. After that time, fertilised eggs were placed in a mesh egg basket suspended within a 10L plastic tank. The tanks containing eggs were filled with filtered and UV-sterilised water from the aquarium system and kept at 12 °C. Each tank was fitted with an air stone to ensure water oxygenation, and approx. 0.5 mL of methylene blue was added as a disinfectant.

Following egg stripping, females were released back into their home tanks, where they continued to be subjected to the original treatment (Control or Experimental) until they produced a third clutch and/or until the end of the experiment (day 67 of the UCSP treatment).

Maternal and egg cortisol analysis

A water sample was collected from each female (n=40) at the time when the second clutch was produced (at least 11 days after the start of the UCSP), to measure maternal baseline cortisol levels following a period of chronic stress exposure. Sample collection, cortisol extraction and quantification are described fully in Chapter 2.

A protocol based on Giesing *et al*. (2011) was used to measure the levels of cortisol deposited by females from both the Control and Experimental groups in their eggs, using a commercial colorimetric competitive cortisol ELISA assay (Enzo Life Sciences) without prior cortisol extraction. Eggs were defrosted on ice and 50 mg of eggs (15-32 eggs) from each clutch were weighed out and placed in an Eppendorf tube containing 200 µL of assay buffer (supplied with the kit). A

disposable tissue grinder was used to homogenise the eggs in buffer. The homogenates were centrifuged at 13.300 rpm for 2 minutes to separate insoluble material, and the supernatant was removed to fresh tubes. Undiluted samples were assayed in duplicate on a 96-wells ELISA plate, according to the manufacturer's instructions, along a set of cortisol standards ranging from 156 to 10,000 pg/mL. Three samples were later diluted in an assay buffer (1:4 and 1:8) and re-assayed due to their cortisol concentration being outside of the standard curve range. The optical density (OD) of each sample was determined using a spectrophotometer (Multiskan Spectrum, Thermo Scientific) and the calculated cortisol concentration was given in ng/mL. The duplicates were used to calculate the intra-assay coefficient of variation (CV). A reference sample, containing pooled egg homogenates from six randomly selected stock fish, was run at the beginning and at the end of each plate to determine the inter-assay CV. Mean values of the intra-assay CV and inter-assay CV across three plates were 8.8% and 13% respectively. Samples with high variability between duplicates (CV >20%, n=5 out of 83 assayed samples) were excluded from further analysis.

Clutch and egg characteristics

Eggs were counted from the photographs to determine clutch size. The mean mass of a single egg was obtained by dividing total clutch mass by the number of eggs in a clutch. Mean egg volume was measured using ImageJ processing software (Rasband, 2016) with the Cell Magic Wand plugin tool developed by Theo Walker. The millimetre paper included in the photograph was used to calibrate the scale and the Cell Magic Wand tool was used to outline and measure the diameter of each egg (in mm) using the "dark cells on light background" setting. The diameter was then converted to egg volume using the formula volume = $4/3\pi r^3$, where r = ½ x diameter.

Development time, hatching rate and fry size at hatching

Egg baskets were checked on a daily basis for hatching, from approximately day 8 post-fertilisation. The date of hatching was recorded as the day on which the last fry hatched. Upon hatching, all fry were removed from the tank into a 55mm Petri dish using a disposable Pasteur pipette, counted and photographed on a lightbox with a piece of millimetre paper. Temperature-corrected development time of each clutch, expressed as the number of Accumulated

Thermal Units (ATU), was determined from aquarium temperature records using the following formula: ATU = number of days between fertilisation and hatching*average temperature. Hatching rate was calculated by dividing the number of hatched fry by the number of fertilised eggs. Mean fry size at hatching was measured using ImageJ. The millimetre paper included in the photograph was used to calibrate the scale, and the standard length of up to 10 fry per clutch was measured using a built-in straight line tool in ImageJ.

Data analysis

The MyAssays online data analysis tool (MyAssays Ltd.) was used to calculate egg cortisol concentrations from optical densities, using the Four Parameter Logistic Curve as per the kit manufacturer's instructions. Statistical analyses of egg cortisol were restricted to the females that produced two or three clutches across the breeding season (to allow for comparison between successive clutches produced by a female across the breeding season), that produced enough eggs per clutch to allow for egg cortisol assay, and for which the information on baseline cortisol level was available. This yielded a sample size of 12 Control females (Clutch 1 n=12, Clutch 2 n=12, Clutch 3 n=9) and 7 Experimental females (Clutch 1 n=5, Clutch 2 n=5, Clutch 3 n=6; sample size variation in this and later analyses was due to the missing data for some of the variables). A Linear Mixed Model (LMM) was used to analyse potential differences in egg cortisol levels between the two treatment groups. The fixed factors included in the initial model were treatment group (categorical variable), clutch number (categorical variable, being either the 1st, 2nd or 3rd clutch of a female), female post-stripping (somatic) mass (covariate), baseline cortisol level of a female (covariate), and interaction terms treatment group*clutch number and treatment group*female cortisol. In this and all subsequent models Tank ID was included as a random factor to control for the potential lack of independence between the fish in a tank; Fish ID was included as a second random factor here and in later analyses that used multiple measurements from the same female. For all models, non-significant terms were removed by backwards selection. Julian date of fertilisation was not included in this or later models due to its correlation with clutch number.

A LMM with Poisson distribution was used to analyse clutch size in the two treatment groups. The analysis was restricted to females that produced two or

three clutches across the breeding season, yielding a sample size of 20 Control females (Clutch 1 n=19, Clutch 2 n=20, Clutch 3 n=15) and 19 Experimental females (Clutch 1 n=19, Clutch 2 n=19, Clutch 3 n=14). Initially, female cortisol concentration and egg cortisol concentration were included as fixed factors in the model (yielding a sample size that was the same as in the egg cortisol data analysis), but these were both shown to be non-significant and were not included in further analyses so as to increase the sample size. The fixed factors included in the initial model were treatment group (categorical variable), clutch number (categorical variable), female somatic mass (covariate) and the interaction term treatment group*clutch number.

Similarly, female cortisol and egg cortisol were not included in the comparisons of single egg mass and egg volume across the two treatment groups, which used LMMs on the same sample size as previously, with the exception of one clutch (Experimental group, Clutch 1) for which no egg mass data were collected. The fixed factors included in the initial models were treatment group (categorical variable), clutch number (categorical variable), clutch size (covariate) and interaction terms: treatment group*clutch number and treatment group*clutch size. Single egg mass values were log-transformed to achieve a normal distribution of the residuals.

A LMM with a negative binomial distribution was used to analyse the potential differences in the temperature-corrected development time (as ATU) of embryos from the two treatment groups. Missing data for some of the variables and failure of 19 out of 125 clutches to hatch restricted the sample size, reducing the statistical power of the model. To account for this, females that produced only one clutch were included in the analysis of the development time, yielding the sample size of 20 Control females (Clutch 1 n=18, Clutch 2 n=11, Clutch 3 n=10) and 21 Experimental females (Clutch 1 n=15, Clutch 2 n=6, Clutch 3 n=7). The fixed factors included in the initial model were treatment group (categorical variable), clutch number (categorical variable), female somatic mass (covariate), egg cortisol level (covariate), egg volume (covariate) and interaction terms: treatment group*clutch number, treatment group*female mass, treatment group*egg cortisol level and treatment group*egg volume.

Hatching rates of eggs from the two treatment groups were analysed by a LMM, with the same sample size as used in the analysis of development time. The

fixed factors included in the initial model were treatment group (categorical variable), clutch number (categorical variable), female cortisol level (covariate) and egg cortisol level (covariate), as well as the interaction terms between all of the covariates and treatment group, and all of the covariates and clutch number. The response variable (hatching rate) was arcsine-transformed.

A LMM was used to analyse the size at hatching, restricting the sample size to fry from females for which the information on fry size was available from at least two clutches. This produced a total of 17 Control females (Clutch 1 n=16, Clutch 2 n=14, Clutch 3 n=13) and 15 Experimental females (Clutch 1 n=14, Clutch 2 n=13, Clutch 3 n=11). The fixed factors included in the initial model were treatment group (categorical variable), clutch number (categorical variable), female mass (covariate), egg volume (covariate) and development time (covariate), as well as the interaction terms between all of the covariates and treatment group, and all of the covariates and clutch number. Initially, Tank ID and Fish ID were included as random factors. However, the variance of the random effects was estimated as 0 (resulting in a singular model fit) and they were thus dropped without affecting model estimates. Female cortisol concentration and egg cortisol concentration were also initially included as fixed factors in the model, but these were found to be non-significant and were not included in further analysis to increase the sample size.

All statistical analyses were performed in R statistical software (version 3.4.3, R Development Core Team), with LMMs fitted using the "lme4" package in R (Bates *et al.*, 2015). The degrees of freedom were estimated by Satterthwaite approximation (for normally distributed data) and Laplace approximation (for Poisson and negative binomial distributed data). P values were obtained from t-statistics or z-statistic using the "lmerTest" package, with an alpha level of 0.05 (Kuznetsova *et al.*, 2017).

Results

Cortisol concentration in eggs

Stress-exposed female three spined-sticklebacks did not deposit more cortisol in their eggs than Control females (Table 3.1). There was, however, a pattern across the sequence of clutches produced by females over the breeding season, with middle clutches (Clutch 2) having significantly higher cortisol levels than early (Clutch 1) or late (Clutch 3) clutches, irrespective of the treatment group (Table 3.1 and Figure 3.1). Neither female mass nor female baseline cortisol level had an effect on the level of cortisol in eggs (Figure 3.2, Table 3.1).

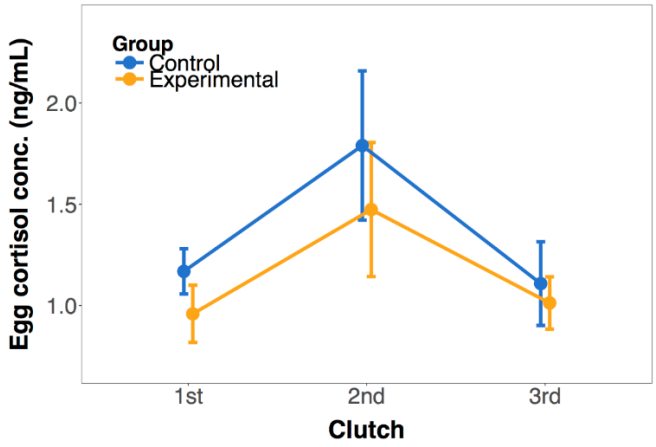

Figure 3.1 Differences in egg cortisol levels (expressed as ng/mL) in three successive clutches produced across the breeding season by three-spined stickleback females from Control (n=12; Clutch 1 n=12, Clutch 2 n=12, Clutch 3 n=9; blue) and Experimental (n=7; Clutch 1 n=5, Clutch 2=5, Clutch 3=6; orange) treatment groups. The error bars represent Standard Error of the Mean (s.e).

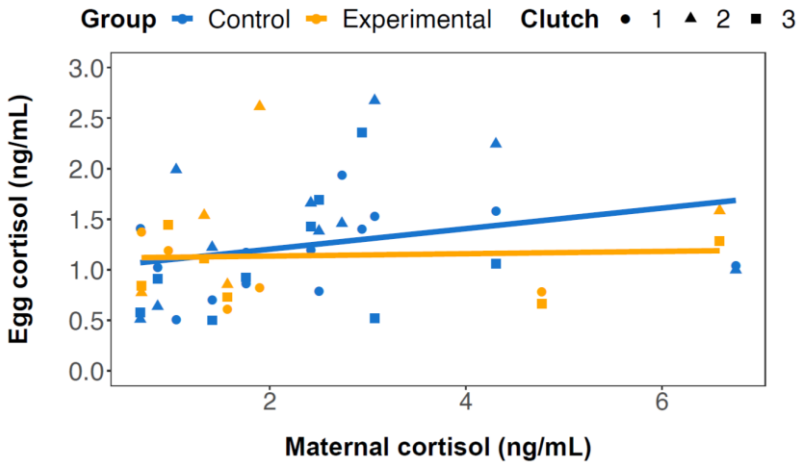

Figure 3.2 The relationship between maternal baseline cortisol level and the level of cortisol in eggs (in ng/mL) of females from Control (n=12; Clutch 1 n=12, Clutch 2 n=12, Clutch 3 n=9; blue) and Experimental (n=7; Clutch 1 n=5, Clutch 2=5, Clutch 3=6; orange) treatment groups. The regression lines were fitted using linear smoothing.

Table 3.1 Summary of Linear Mixed Models (LMM) used to analyse the differences in egg cortisol levels in three successive clutches produced by Control and Experimental female three-spined sticklebacks during the breeding season. The reference Treatment was the Control group. Female ID and tank ID were included in the models as random factors. Control group n=12 females, Experimental group n=7. Treatment group, Clutch and all significant terms (indicated with p-values in underlined and bold font) were retained in the final model.

Model terms	Effect on egg cortisol levels				
	Est.	s.e.	df	t value	p value
Treatment group	-0.189	0.276	15.11	-0.685	0.504
Clutch 2	0.550	0.213	33.16	2.577	**0.015**
Clutch 3	-0.036	0.222	33.34	-0.164	0.871
Female mass	0.305	0.402	40.10	0.756	0.454
Female cortisol level	0.055	0.073	17.24	0.744	0.467
Treatment group*Clutch 2	-0.028	0.498	30.70	-0.056	0.955
Treatment group*Clutch 3	0.228	0.496	30.20	0.460	0.648
Treatment group*Female cortisol	-0.064	0.163	13.81	-0.393	0.700

Clutch and egg characteristics

Preliminary analysis revealed the presence of four clutches with abnormal residuals (difference of more than 3 standard deviations from the mean), which affected the distribution of model residuals. These outliers were removed, and data re-analysed, with the results of the refined analysis being qualitatively similar to the original analysis that included the full dataset (shown in Appendix 3). The average clutch size of females exposed to a period of UCSP did not differ from that of Control females (Figure 3.3, Table 3.2), but there were differences in the size of successive clutches produced over the breeding season, with Clutch 2 being significantly smaller than Clutch 1 and Clutch 3 (Table 3.2). In addition, clutch size showed a significant positive correlation with female mass (Figure 3.4).

Stress-exposed mothers had a tendency to produce lighter eggs than Control mothers, but this effect was not statistically significant. There was a significant increase in the mass of an egg throughout the breeding season, with eggs from later clutches being heavier (Figure 3.3, Table 3.2). In addition, egg mass was influenced by a significant interaction between treatment group and clutch size: whilst overall there was a strong negative relationship between egg mass and clutch size across both groups analysed together, in the Experimental group individual eggs were heavier in larger clutches (Figure 3.4, Table 3.2).

Egg volume was independent of the treatment group. A tendency was observed for egg volume to increase in Clutch 2 and Clutch 3, but this was marginally insignificant (Figure 3.3, Table 3.2). There was also a negative effect of clutch size on egg volume in both treatment groups (Figure 3.4 and Table 3.2).

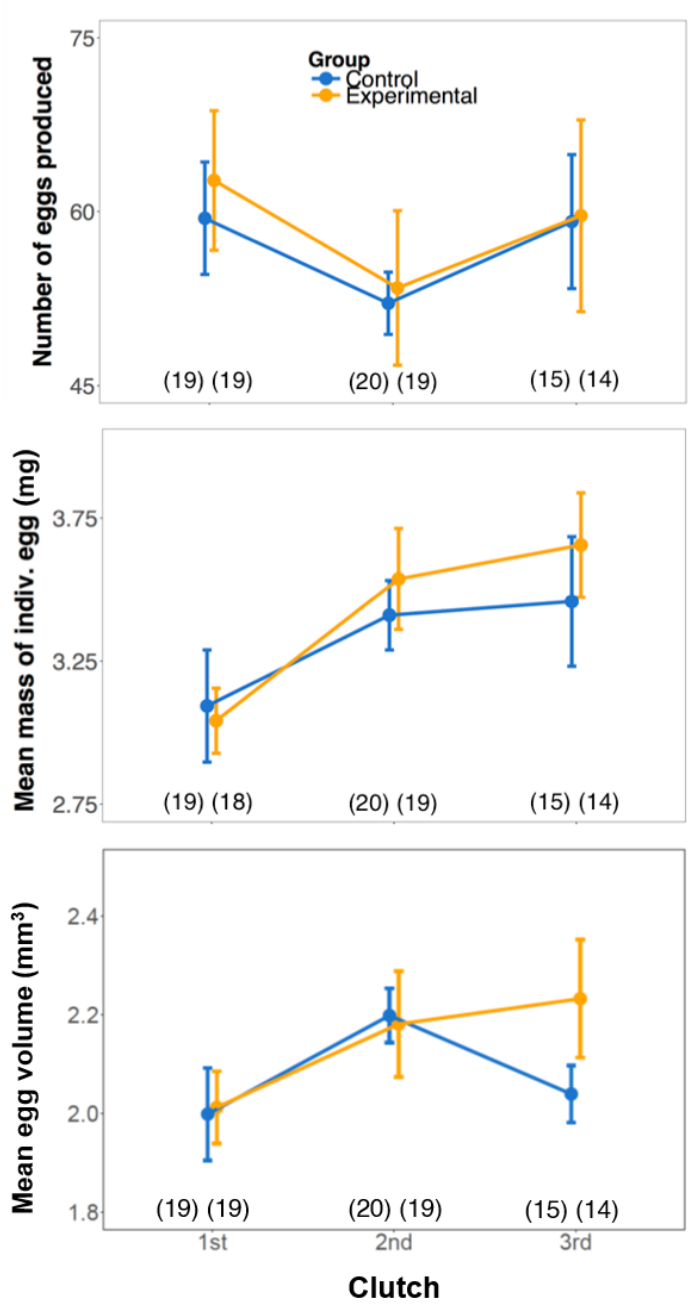

Figure 3.3 The differences in clutch size (as mean number of eggs per clutch, top panel), mean mass of an individual egg (in mg, middle panel) and egg volume(in mm^3), bottom panel) in three successive clutches produced across the breeding season by female three-spined sticklebacks from Control (n=20; blue) and Experimental (n=19; orange) treatment groups. Sample sizes for each clutch are given in brackets. The error bars represent Standard Error of the Mean (s.e).

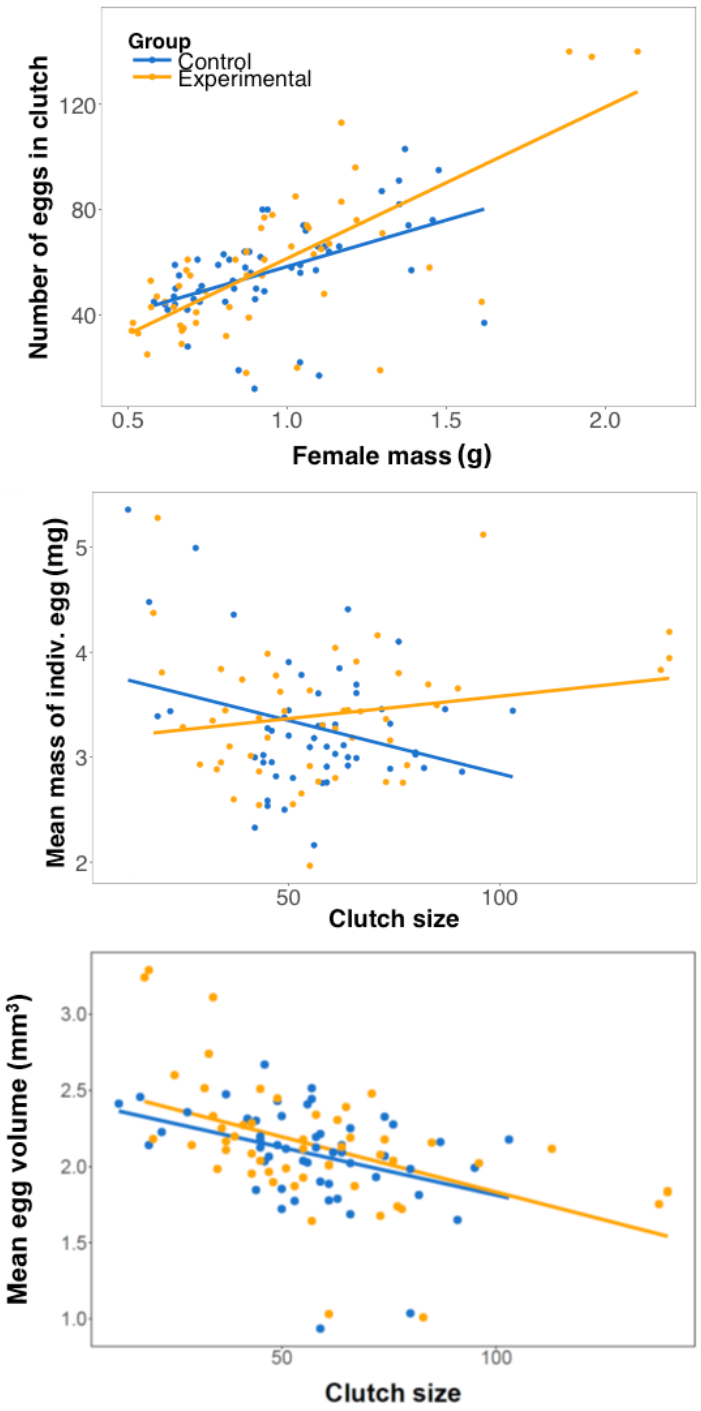

Figure 3.4 The relationship between female three-spined stickleback mass (in grams) and clutch size (top panel), between clutch size and mean mass of an individual egg (in mg, middle panel), and between clutch size and mean egg volume (in mm^3, bottom panel) in females from Control (n=20; blue) and Experimental (n=19; orange) treatment groups. The regression lines were fitted using linear smoothing. For clarity, individual egg mass data were standardised for an average clutch size.

Table 3.2 Summary of Linear Mixed Models (LMM) used to analyse the differences in clutch size, single egg mass (in mg; log-transformed) and egg volume (in mm^3) in three successive clutches produced by Control and Experimental female three-spined sticklebacks during the breeding season. Values give estimates ± s.e. The reference Treatment in all models was the Control group. Female ID and tank ID were included in the models as random factors. Control group n=20 females (Clutch 1 n=19, Clutch 2 n=20, Clutch 3 n=15), Experimental group n=19 (Clutch 1 n=19, Clutch 2 n=19, Clutch 3 n=14). Treatment group, Clutch and all significant terms (indicated with p-values in underlined and bold font) were retained in the final models.

Model terms	**Effect on clutch size**	**Effect on log egg mass**	**Effect on egg volume**
Treatment group	-0.024 ± 0.069, z=-0.355, p=0.722	-0.158 ± 0.096, $t_{1,63.43}$=-1.652, p=0.103	0.061 ± 0.076, $t_{1,36.99}$=0.565, p=0.430
Clutch 2	-0.101 ± 0.031, z=-3.260, **p=0.001**	0.087 ± 0.034, $t_{2,63.58}$=2.546, **p=0.013**	0.128 ± 0.066, $t_{2,69.10}$=1.930, p=0.058
Clutch 3	-0.041 ± 0.033, z=-1.234, p=0.217	0.138 ± 0.038, $t_{2,69.19}$=3.659, **p<0.001**	0.122 ± 0.071, $t_{2,71.70}$=1.712, p=0.091
Treatment group*Clutch 2	0.087 ± 0.062, z=1.406, p=0.160	0.052 ± 0.069, $t_{2,61.96}$=0.760, p=0.450	-0.047 ± 0.131, $t_{2,65.11}$=-0.361, p=0.719
Treatment group*Clutch 3	-0.023 ± 0.067, z=-0.344, p=0.731	0.084 ± 0.076, $t_{2,67.41}$=1.106, p=0.272	0.149 ± 0.143, $t_{2,70.18}$=1.037, p=0.303
Female mass	0.181 ± 0.032, z=5.685, **p<0.001**	NA	NA
Treatment group*Mass	NA	NA	NA
Clutch size	NA	-0.007 ± 0.001, $t_{1,80.27}$=-5.447, **p<0.001**	-0.007 ± 0.001, $t_{1,65.97}$=-4.636, **p<0.001**
Treatment group*clutch size	NA	0.004 ± 0.002, $t_{1,70.34}$=2.219, **p=0.030**	0.000 ± 0.003, $t_{1,73.77}$=0.204, p=0.837

Development time, hatching rate and fry size at hatching

The temperature-corrected development time was the same in eggs of stress-exposed and Control females and was independent of the maternal clutch number (Figure 3.5, Table 3.3), cortisol concentration in eggs and egg volume (Table 3.3). Larger stress-exposed females produced eggs with a shorter development time, whilst the eggs of larger Control females took longer to hatch (Figure 3.6, Table 3.3).

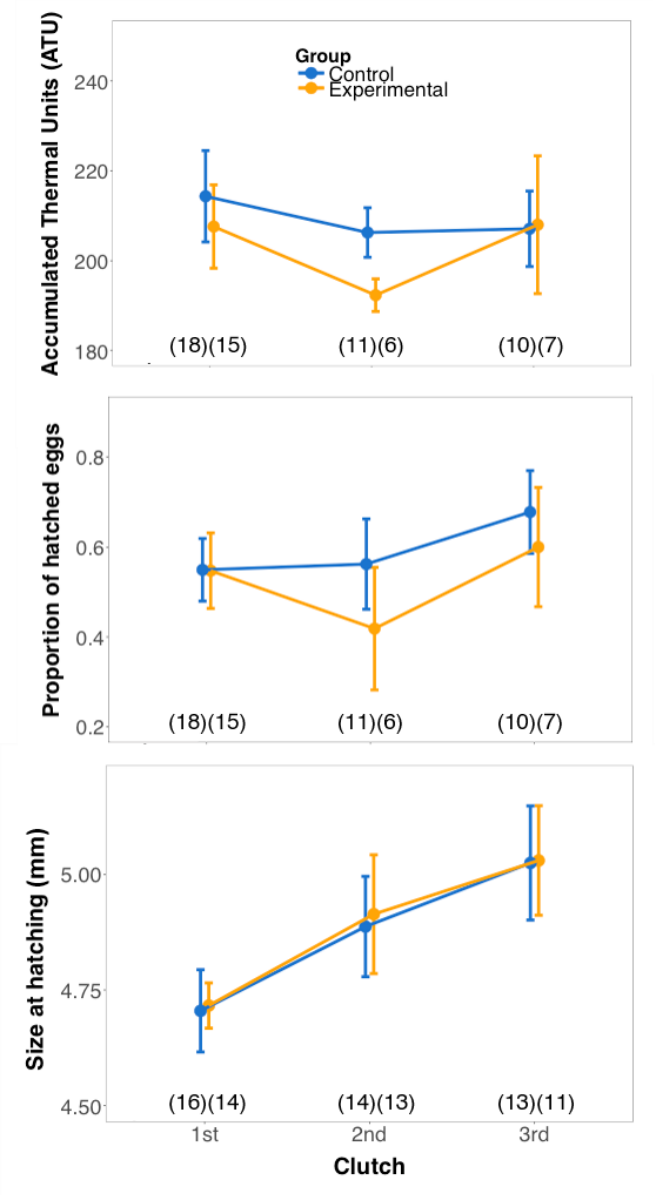

Figure 3.5 The differences in development time, expressed as Accumulated Thermal Units (no. of days between fertilisation and hatching, multiplied by average temperature), hatching rate (as proportion of eggs that hatched) and fry size at hatching (length in mm) in three successive clutches produced across the breeding season by female three-spined sticklebacks from Control (n=20; blue) and Experimental (n=21; orange) treatment group. Sample sizes for each clutch are given in brackets. The error bars represent Standard Error of the Mean (s.e).

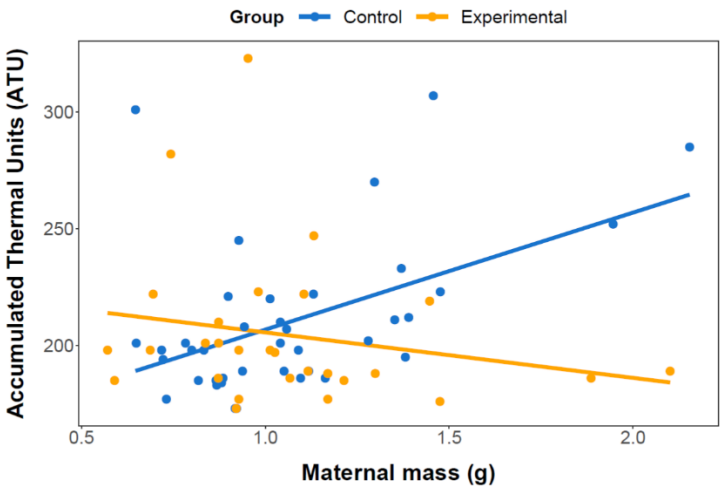

Figure 3.6 The relationship between temperature-corrected development time, expressed as Accumulated Thermal Units (no. of days between fertilisation and hatching multiplied by average temperature) and female somatic mass (in g) in Control (n=20; blue) and Experimental (n=21; orange) treatment group. The regression lines were fitted using linear smoothing.

Table 3.3 Summary of Linear Mixed Model (LMM) used to analyse the differences in development time (as Accumulated Thermal Units, no. of days between fertilisation and hatching * average temperature) in three successive clutches produced by Control and Experimental female three-spined sticklebacks during the breeding season. The reference Treatment was the Control group. Female ID and tank ID were included in the models as random factors. Control group n=20 females (Clutch 1 n=18, Clutch 2 n=11, Clutch 3 n=10), Experimental group n=21 (Clutch 1 n=15, Clutch 2 n=6, Clutch 3 n=7). Treatment group, Clutch and all significant terms (indicated with p-values in underlined and bold font) were retained in the final model.

Model terms	Effect on development time (ATU)			
	Est.	s.e.	z value	p value
Treatment group	-0.018	0.037	-0.492	0.623
Clutch 2	-0.037	0.039	-0.960	0.337
Clutch 3	-0.019	0.038	-0.503	0.615
Treatment *Clutch2	-0.053	0.085	-0.619	0.536
Treatment *Clutch 3	0.011	0.073	0.158	0.875
Female mass	0.074	0.024	3.112	**0.002**
Treatment*Female mass	-0.111	0.035	-3.154	**0.002**
Egg cortisol level	0.039	0.024	1.641	0.101
Treatment*Egg cortisol	-0.087	0.052	-1.650	0.099
Egg volume	-0.012	0.017	-0.701	0.483
Treatment*Egg volume	0.058	0.036	1.640	0.101

There was no difference in hatching rate between the treatment groups, but there was an increase in hatching rate throughout the breeding season, with the late clutches (Clutch 3) having a greater hatching success (Figure 3.5, Table 3.4). There was also an indication of a relationship between treatment group and female cortisol levels, with higher maternal cortisol levels resulting in a lower hatching rate in Experimental but not in Control females, but this was marginally insignificant.

Table 3.4 Summary of the Linear Mixed Model (LMM) used to analyse the differences in hatching rate (as proportion of hatched eggs) in three successive clutches produced by Control and Experimental female three-spined sticklebacks during the breeding season. The reference Treatment was the Control group. Female ID and tank ID were included in the models as random factors. Control group n=20 females (Clutch 1 n=18, Clutch 2 n=11, Clutch 3 n=10), Experimental group n=21 (Clutch 1 n=15, Clutch 2 n=6, Clutch 3 n=7). Treatment group, Clutch and all significant terms (indicated with p-values in underlined and bold font) were retained in the final model.

Model terms	**Effect on hatching rate**				
	Est.	**s.e.**	**df**	**t value**	**p value**
Treatment group	0.227	0.223	28.22	1.019	0.317
Clutch 2	-0.035	0.096	32.46	2.546	0.718
Clutch 3	0.266	0.105	35.02	2.539	**0.016**
Treatment group*Clutch 2	0.090	0.063	21.18	1.416	0.171
Treatment group*Clutch 3	0.093	0.077	23.80	1.218	0.235
Female cortisol level	0.116	0.060	24.11	0.193	0.848
Treatment*Female cortisol	-0.155	0.080	28.17	1.927	0.064
Clutch 2*Female cortisol	0.097	0.061	23.14	1.592	0.125
Clutch 3*Female cortisol	0.103	0.074	26.00	1.388	0.177

The mean size at hatching of offspring from stress-exposed females did not differ significantly from that of offspring from Control females, but fry hatching from eggs laid later in the season (Clutch 3) were significantly larger than those from early and middle clutches (Figure 3.5, Table 3.5). I also observed a relationship between fry size at hatching, treatment group and maternal mass: whilst in the Control group heavier mothers produced offspring that were larger at hatching, the opposite was true in the Experimental group (Figure 3.7). In addition, there was a significant interaction between clutch number and egg volume, with a significant positive correlation between egg volume and fry size at hatching in Clutch 2 only (Figure 3.7, Table 3.5).

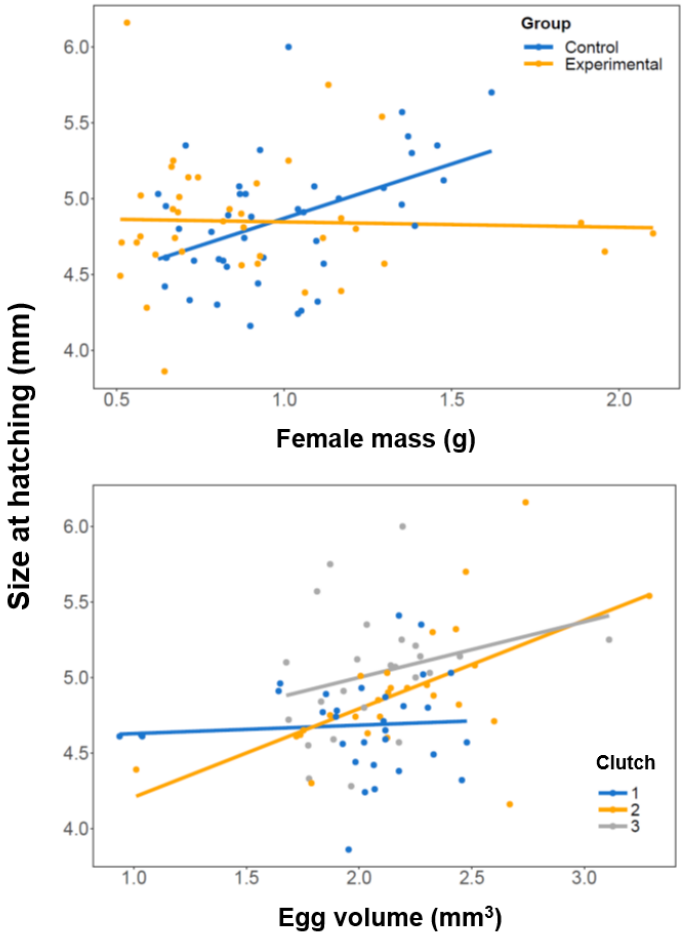

Figure 3.7 The relationship between fry size at hatching and female three-spined stickleback mass in Control (n=17; blue) and Experimental (n=15; orange) treatment group (top panel), and between fry size at hatching and egg volume in three successive clutches (Clutch 1, n=30, blue; Clutch 2, n=27, orange; Clutch 3 n=24, grey) produced across the breeding season (bottom panel). The regression lines were fitted using linear smoothing.

Table 3.5 Summary of the Linear Model (LM) used to analyse the differences in fry size at hatching (in mm) in three successive clutches produced by Control and Experimental female three-spined sticklebacks during the breeding season. The reference Treatment was the Control group. Control group n=17 females (Clutch 1 n=16, Clutch 2 n=14, Clutch 3 n=13), Experimental group n=15 (Clutch 1 n=14, Clutch 2 n=13, Clutch 3 n=11). Treatment group, Clutch and all significant terms (indicated with p-values in underlined and bold font) were retained in the final model.

Model terms	**Effect on fry size at hatching**
Treatment group	0.021 ± 0.078, $t_{1,64}$=0.274, p=0.785
Clutch 2	0.154 ± 0.094, $t_{2,64}$=1.652, p=0.103
Clutch 3	0.334 ± 0.095, $t_{2,64}$=3.513, **p<0.001**
Treatment group*Clutch 2	0.156 ± 0.196, $t_{2,64}$=0.800, p=0.427
Treatment group*Clutch 3	-0.050 ± 0.198, $t_{2,64}$=-0.252, p=0.802
Female mass	0.198 ± 0.067, $t_{1,64}$=2.959, **p=0.004**
Treatment*Female mass	-0.182 ± 0.083, $t_{1,64}$=-2.187, **p=0.032**
Clutch 2*Female mass	-0.041 ± 0.089, $t_{2,64}$=-0.457, p=0.649
Clutch 3*Female mass	0.033 ± 0.102, $t_{2,64}$=0.321, p=0.749
Egg volume	0.002 ± 0.063, $t_{1,64}$=0.028, p=0.978
Treatment*Egg volume	-0.000 ± 0.091, $t_{1,64}$=-0.010, p=0.992
Clutch 2*Egg volume	0.198 ± 0.088, $t_{2,64}$=2.246, **p=0.028**
Clutch 3*Egg volume	0.151 ± 0.111, $t_{1,64}$=1.350, p=0.181
Development time (ATU)	0.002 ± 0.001, $t_{1,64}$=1.657, p=0.102
Treatment group*ATU	0.001 ± 0.003, $t_{1,64}$=0.313, p=0.755
Clutch 2*ATU	-0.006 ± 0.003, $t_{2,64}$=-1.719, p=0.090
Clutch 3*ATU	0.000 ± 0.003, $t_{2,64}$=0.072, p=0.943

Discussion

Egg cortisol levels

This study found no correlation between maternal and egg cortisol levels. This is contrary to the evidence from studies on a wide range of taxa, including humans (Baibazarova *et al.*, 2013), Antarctic fur seals (Meise *et al.*, 2016), Japanese quail (Okuliarova *et al.*, 2010), European starlings (Love *et al.*, 2005b), barn swallows (Saino *et al.*, 2005) and eastern fence lizards (Ensminger *et al.*, 2018), which show that levels of glucocorticoids present in maternal plasma or tissues are reflected in GC levels of their ova and developing embryos. As in other vertebrates, a correlation between maternal and egg cortisol levels has also been reported in various fish species. Coho salmon exposed to daily chasing regimen had elevated plasma cortisol levels, which positively correlated with egg cortisol (Stratholt *et al.*, 1997). Analogous results were reported by Sierra-Flores *et al.* (2015) in female Atlantic cod, who used anthropogenic noise as a relatively mild stressor. Giesing *et al.* (2011) did not measure maternal cortisol levels, but found a significant relationship between female exposure to predators and elevated egg cortisol levels in three-spined stickleback. Simulating stressful conditions by implanting females with cortisol has led to increase in egg cortisol e.g. in Atlantic salmon (Eriksen *et al.*, 2006) and Atlantic cod (Kleppe *et al.*, 2013), and to elevated ovarian cortisol in European sturgeon (Poursaeid *et al.*, 2012). However, mechanisms have been identified that allow mothers to buffer their developing offspring from excess cortisol (Benediktsson *et al.*, 1997), with limited evidence that in fish these mechanisms may act at the level of the ovary to prevent cortisol deposition during egg production (Faught *et al.*, 2016). Such maternal buffering could therefore explain why egg cortisol was independent of maternal cortisol in the present study.

In addition to the lack of correlation between maternal and egg cortisol concentrations, exposure of females to chronic stressors did not result in higher cortisol levels in their eggs. This raises a question about the mechanistic cause of the observed lack of a maternal stress effect on glucocorticoids *in ovo*. It has been previously proposed that certain molecular mechanisms are responsible for the transport of excess cortisol out of the eggs of fish post-fertilisation (Paitz *et al.*, 2015, Paitz *et al.*, 2016), which could also explain the lack of any effect of maternal cortisol treatment on embryo cortisol levels observed by Redfern *et al.*

(2017) in largemouth bass. However, in my study cortisol levels were measured in unfertilised eggs and therefore mechanisms intrinsic to embryos are unlikely to have affected the findings. Instead, I propose two possible mechanisms driving the lack of an effect of maternal stress exposure on egg cortisol levels. Firstly, protracted exposure to stressful conditions may lead to exhaustion of the HPI axis (Hontela *et al.*, 1997, Barton, 2002), resulting in reduced availability of cortisol. This would explain the lack of increase in baseline cortisol of stress-exposed females, observed in Chapter 2 of this thesis. This is congruent with the study on brook trout, where neither cortisol supplementation nor handling caused an elevation of maternal or egg cortisol levels (Cortez Cortez Ghio *et al.*, 2016). Secondly, an expression of glucocorticoid-inactivating enzymes in ovaries was observed in zebrafish (Faught *et al.*, 2016), which may act as a mechanism of maternal buffering against excess cortisol and its potential maladaptive consequences for offspring phenotype. Notably, despite a common preconception that cortisol levels are correlated with fish size (Scott and Ellis, 2007), and previous evidence that larger three-spined stickleback females deposit a higher concentration of cortisol in eggs (Giesing *et al.*, 2011), my study shows no relationship between egg cortisol and female mass. This provides further ground for consideration of active maternal regulation rather than passive transfer of cortisol into eggs.

Egg cortisol concentrations in this study, which, to the best of my knowledge, is the first one to examine the effects of relatively low intensity, protracted unpredictable stress on egg and offspring phenotype, were independent of maternal stress treatment. To date only Mileva *et al.* (2011) have linked inter-clutch differences in egg cortisol with maternal stress exposure, using the daffodil cichlid. Unlike in my study, where female treatment had no effect on egg cortisol, their study showed a significant interaction between female treatment and seasonal changes in egg cortisol, with eggs of stress-exposed females showing no decline in cortisol levels across the sequence of clutches. It is however important to note that the study of Mileva *et al.* used a single form of repeated, acute stressor (chasing) throughout the experimental period, which may produce a different effect to varied stressors applied in a long-term unpredictable manner.

Notwithstanding, I did identify inter-clutch differences in egg cortisol levels, with clutches produced in the middle of the breeding season having a

significantly higher cortisol content, independent of the female treatment group. Whilst inter-clutch changes in fish gamete size (Daoulas and Economou, 1986, Clarke, 1989, Johnston and Leggett, 2002) and quality (Skaalsvik *et al.*, 2015) have been widely investigated, considerably less is known about seasonal or inter-clutch variation in *in ovo* cortisol levels. It has been shown in birds that GCs in egg yolk differ between clutches and between successive ovulations within a clutch (Love *et al.*, 2008), but the evidence for similar patterns in fish is limited. Skaalsvik *et al.* (2015) reported a decline in egg cortisol in Atlantic halibut over the spawning season, whilst Asian sea bass showed more unpredictable inter-clutch variations (Sampath-Kumar *et al.*, 1995). My study thus contributes to the existing knowledge that cortisol levels in fish eggs vary between the clutches. It was however beyond the scope of my research to examine the mechanisms behind this variation.

Clutch and egg phenotype

In addition to the lack of difference in egg cortisol levels between stress-exposed and control females, the results of my study show no significant effect of female stress treatment on clutch size, or volume and weight of individual eggs. Moreover, I did not detect a relationship between egg cortisol and clutch/egg size. What emerges from my study, and other studies on maternal stress effects using maternal exposure to stressors of various nature and intensity, is the lack of a uniform pattern in which stress-exposed females influence clutch and egg phenotype. For example, Ambon damselfish subjected to crowding (McCormick, 2006), Atlantic cod subjected to anthropogenic noise (Sierra-Flores *et al.*, 2015) and zebrafish exposed to social stress (Jeffrey and Gilmour, 2016) produced the same number of eggs per clutch as controls. Lack of maternal stress effect on clutch and egg size has been reported in other vertebrate species, including Japanese quail (Hayward and Wingfield, 2004) and the eastern fence lizard (Ensminger *et al.*, 2018).

However, there is also extensive evidence that maternal stress treatment does have an effect on clutch and egg phenotype. Simulated risk of nest predation in great tits resulted in smaller clutches (Travers *et al.*, 2010), whilst corticosterone-supplemented zebra finches produced more eggs per clutch than controls (Khan *et al.*, 2016b). Furthermore, McConnachie *et al.* (2012) reported a negative effect of cortisol implants on clutch size in pink salmon. Whilst care

must be taken when interpreting the results of studies using cortisol implants, as the process of implantation has been previously shown to negatively affect egg size in brown trout (Hoogenboom *et al.*, 2011), similar effects have been observed in other contexts. Exposure to stressors in the period leading up to spawning led to a reduction in egg size in daffodil cichlid (Mileva *et al.*, 2011), rainbow trout (Campbell *et al.*, 1992) and eastern mosquitofish (Mukherjee *et al.*, 2014). Unpredictability of the maternal environment has been shown to result in smaller egg size and greater intra-clutch variability in three-spined stickleback (Shama, 2015) and pygmy perch (Morrongiello *et al.*, 2012).

The relationship between stress and female reproductive strategy in terms of investment in clutch and egg size is therefore a complex issue, and the nature of this relationship is likely to depend on interplay of many factors, such as nature and timing of the stressor, maternal condition (Rollinson and Brooks, 2008), quality of the environment (Rollinson and Hutchings, 2013) etc. For example, exposure to stressors can negatively influence female body condition (Hosoya *et al.*, 2007, Leal *et al.*, 2011), and females in poor condition have been shown to alter egg provisioning and produce smaller clutches (Reznick *et al.*, 1996, Warner *et al.*, 2007). Oocyte production and development is a multi-stage process during which eggs accumulate material (e.g. proteins and lipids) of endogenous and exogenous origin (Pelegri, 2003). Some of the stages are more critical to clutch/egg phenotype: for example during the vitellogenesis (yolk formation) eggs increase in size by up to 95% by incorporating proteins of maternal origin (Tyler and Sumpter, 1996). It is therefore reasonable to expect that reduced egg provisioning due to stressful conditions during vitellogenesis will have a negative impact on final egg size, as shown e.g. by Contreras-Sanchez *et al.* (1998). In addition, the rate of oocyte development and atresia can be up- or downregulated in response to environmental conditions (Tyler and Sumpter, 1996, Habibi and Andreu-Vieyra, 2007), resulting in altered clutch size.

The way in which females use resources for egg provisioning further adds to the complexity of the stress-reproduction relationship. Capital breeders provision their eggs with resources acquired prior to the breeding season, whilst income breeders rely on resources acquired during the breeding season (Jonsson, 1997, McBride *et al.*, 2015). Thus the timing of stress-exposure (before or during breeding) can be crucial for determining of the effect of maternal stress on egg phenotype. For example, capital breeders can pay a higher cost of energy

storage (Bonnet *et al.*, 1998), which combined with the energetic demands of stress response may affect the availability of resources for egg provisioning. At the same time, stressful conditions experienced by income breeders during the breeding season can also negatively affect the number and size of eggs if maternal food intake is reduced due to stress.

Despite the lack of an effect of maternal stress exposure on clutch size, individual egg mass or volume, there was variation over the course of the breeding season in reproductive investment by females in both treatments. This was manifested in considerable inter-clutch variation in the number of eggs produced and a significant increase in individual egg mass across successive clutches, as well as a tendency for females to produce larger eggs with each subsequent clutch. My analyses were based on clutch order rather than time of year, but these two factors were highly correlated and so clutch order can be considered as a seasonal effect. My results are contrary to the findings of Castillo-Hidalgo *et al*. (2018), who did not detect seasonal variation in clutch size in the marine fish *Sindoscopus australis,* and Louhi *et al*. (2015), who reported the lack of effect of the timing of spawning on egg size in Atlantic cod and coho salmon. Moreover, my results are inconsistent with those from other taxa; examples from insects (Forsman, 2001) and birds (Krapu *et al.*, 2004) show reductions in clutch size across the breeding season. Suarez *et al*. (2005) studied inter-clutch patterns in clutch size of four Mediterranean lark species and found a significant increase in the size of the middle clutches compared to early and late clutches, which is at odds with my results, where the middle clutch had the smallest mean number of eggs.

It is difficult to speculate about the nature of the inter-clutch variation in clutch size and egg size observed in the present study due to the large numbers of factors that can influence female reproductive strategy. These factors may include hormones (e.g. estrogen, serotonin; Thomas *et al.*, 2008) and nutrients (Bobe and Labbe, 2010) and their role may be complex. For example, dietary factors affected clutch size but not egg size in zebrafish (Markovich *et al.*, 2007) and rainbow trout (Blom and Dabrowski, 1995). A plausible explanation of the increase in egg size across the sequence of clutches is related to the annual nature of the population of origin of the fish used in my study. Lee *et al*. (2012) demonstrated that female sticklebacks from the same source population mostly only reproduced in a single breeding season. Fish utilising this mode of breeding

show strong trade-offs between reproduction and self-maintenance, by maximising their reproductive investment towards the end of the breeding season (Poizat *et al.*, 1999). Since clutch size in the present study did not increase significantly over time, it is possible that the females attempted to increase their fitness by investing resources in quality rather than quantity of their eggs, as seen e.g. in Bell (1999). An increase in egg mass towards the end of the breeding season may also be an example of an anticipatory maternal effect. For instance, when overwinter survival of juveniles depends on their body size and condition, females that experienced unfavourable conditions earlier in life may invest in larger eggs towards the end of the breeding season in order to provide their offspring with survival advantage (e.g. Kim *et al.*, 2017).

As expected based on previous evidence from fish and other vertebrates, clutch size in my study was positively correlated with female mass (in den Bosch and Bout, 1998, Vallin and Nissling, 2000, Smalas *et al.*, 2017, but see the metaanalysis of Neuheimer *et al.*, 2015 who found no relationship between maternal and offspring mass in marine teleost fishes). I also observed a negative relationship between clutch size and egg size; this is expected from optimal egg size theory, which predicts a trade-off between egg size and clutch size, when resources are limiting. It also predicts that additional resources above the minimum required to produce one viable egg will be preferentially invested into increasing clutch size rather than egg size (Parker and Begon, 1986, Blackburn, 1991, Christians, 2002, Einum and Fleming, 2002). In my study, the trade-off between clutch size and egg volume was independent of maternal treatment, but exposure of females to a period of chronic stress altered their reproductive strategy in terms of individual egg mass. Whilst in the Control group larger clutches consisted of lighter eggs, as predicted by the theory, the opposite was observed in the Experimental group. I therefore propose that stress-exposed females that had sufficient reserves attempted to maximise their fitness by increasing their clutch size, but also attempted to program their offspring for a future stressful environment by investing in egg quality, indicated by the increased mass of individual eggs. It is unclear why maternal stress treatment affected the relationship between clutch size and egg mass but not between clutch size and egg volume; this question could be answered by analysing factors such as egg composition and nutritional content, which was beyond the scope of this study.

Developmental trajectories

The results of the present study suggest that there is no straightforward relationship between maternal exposure to low intensity chronic stress and offspring developmental trajectories in three-spined sticklebacks. I did not detect a direct effect of maternal treatment on offspring development time, hatching rate or fry size at hatching. The development time was not correlated with cortisol levels in eggs, regardless of the maternal treatment. This is congruent with the evidence from previous studies, e.g. on chinook salmon (Capelle *et al.*, 2016) and Mallee dragon (Uller *et al.*, 2009), which found no differences in developmental rates between glucocorticoid-treated and untreated eggs. Similarly, Stratholt *et al.* (1997) reported an elevation of egg cortisol following the exposure of female coho salmon to stressors, but this elevation had no effect on the development of the resulting embryos.

Despite the lack of a direct effect of maternal stress exposure on offspring development time, I observed a modification of the relationship between maternal mass and offspring development, resulting from chronic exposure to stressors. Generally, the availability of resources that the mother can invest in developing eggs and embryos depends on her size and condition (Ronget *et al.*, 2018). I therefore expected heavier mothers to allocate more resources to their eggs, resulting in higher egg quality and faster development. However, this reduction in the time taken for eggs to hatch with increasing maternal mass was only evident in stress-exposed females, which also had a tendency to produce lighter eggs. As shown by Self *et al.* (2018) in rainbow trout, smaller eggs can have a reduced development time, leading to smaller larval size at hatching, which is offset by increased growth rate post-hatching. Moreover, in my study development time was positively correlated with egg cortisol. It has been previously shown that elevated cortisol in fish eggs can result in a higher metabolic rate (Sloman, 2010, Giesing *et al.*, 2011), which in turn shortens development time (DiMichele and Powers, 1984). It is therefore possible that females in a stressful and unpredictable environment adjust offspring developmental trajectories to reduce development time; in a stable environment it would be more beneficial in fitness terms to produce eggs that take longer to develop but result in larger size at hatching. I also observed an effect of maternal stress exposure on the relationship between egg size (as egg volume) and development time. It has been previously shown that

developmental rate is related to egg size in birds (Wilson, 1991) and fish (Pepin *et al.*, 1997). However, here a significant relationship was due to four clutches with unusually small eggs, and so it is not clear whether this is a true effect.

Subjecting females to a period of chronic stress altered the interrelation between maternal mass and fry size at hatching. Large and heavy females are often in better condition and therefore have more resources available for investment in their developing offspring (Ronget *et al.*, 2018); therefore size of fry or larvae at hatching tends to increase with increasing maternal size (e.g. Kindsvater *et al.*, 2012, O'Dea *et al.*, 2015). There is also a well documented, but not clearly conclusive, effect of egg GCs (either through maternal stress exposure or exogenous GCs *in ovo*) on hatching size. For example, maternal stress/elevated egg GCs resulted in smaller size at hatching in Ambon damselfish (McCormick, 1999) and Atlantic salmon (Eriksen *et al.*, 2006), and larger size at hatching in eastern mosquitofish (Mukherjee *et al.*, 2014). No effect of GCs on hatch size was detected in three-spined sticklebacks (Giesing *et al.*, 2011), eastern fence lizards (Ensminger *et al.*, 2018), Japanese quail (Marasco *et al.*, 2012) or yellow-legged gulls (Rubolini *et al.*, 2005). In addition, the shape of this relationship can be sex-specific (Love *et al.*, 2005b, Uller *et al.*, 2009) and differ even in closely related species (Sopinka *et al.*, 2016b). In my study, I observed the expected linear increase in fry size at hatching with maternal mass in females from the Control group, but the size at hatching of fry from stress-exposed mothers showed a negative correlation with maternal mass. It is unclear why large female sticklebacks adjust their reproductive strategy when facing stressful conditions to produce offspring that are smaller, despite potentially having more resources available to produce larger fry. However, as with the development time, this may be a strategy to increase their own and their offspring's fitness in a stressful and unpredictable environment.

In addition to the effect of stress exposure on female reproductive strategy, I also observed a marked change across the breeding season in hatching rate and fry size at hatching, with the later clutches having a significantly higher hatching success and producing larger fry. Inter-clutch differences in developmental trajectories in fish are rarely investigated, particularly in the context of maternal stress. McCormick (1999) identified an inter-clutch variation in Ambon damselfish size at hatching following cortisol immersion, but the effect was evident in only one out of six clutches and was dose-dependent. A previous study

of three-spined sticklebacks found that maternal stress exposure had no effect on hatch size, but there was an upward trend throughout the breeding season (Giesing *et al.*, 2011). Similarly, in my study the increase in hatching rate and hatch size was irrespective of maternal treatment and therefore unlikely to be driven by exposure of females to chronic stress. Instead, I propose that females in this annual stickleback population (Lee *et al.*, 2012) attempt to maximise their lifetime reproductive success by investing more in the offspring produced towards the end of their reproductive lifespan. In this period, the trade-off between self-maintenance and reproduction becomes less crucial than early in the season (Poizat *et al.*, 1999) and the females can invest more into their offspring to provide them with an overwinter survival advantage (Kim *et al.*, 2017).

In conclusion, the analyses of the effects of long-term, unpredictable chronic stress on female reproductive strategy and early developmental trajectories of their offspring show a complex relationship between these factors. Maternal stress exposure did not affect egg phenotype and offspring development in a straightforward way. Instead, it reshaped the correlations between maternal and clutch parameters in a way that suggests there may be adaptations for maximising maternal and offspring fitness in a stressful and unpredictable environment. However, there is only very weak evidence that these adaptations are driven by cortisol and thus other factors, e.g. nutritional, must be taken into account when interpreting their significance. Furthermore, I observed inter-clutch variations in clutch phenotype and early offspring development, which were consistent with maximisation of reproductive output in an annual species but were independent of maternal treatment. Hence, these results do not support my initial proposal that chronic maternal stress has a differential effect on successive clutches produced by females across a single breeding season.

Chapter 4: The effect of chronic maternal stress on offspring survival and growth

Abstract

If experienced in the period leading up to gamete development and spawning, maternal stress may alter the survival and post-natal developmental trajectories of the offspring. Whilst acute stress is often associated with adaptive maternal programming, the consequences of chronic exposure to environmental and husbandry stressors are less clear in terms of offspring survival and growth. Particularly, little is known about the impact of chronic maternal stress on survival and growth of offspring from successive clutches produced over a single breeding season.

To address these questions, I exposed female three-spined sticklebacks to simulated chronic environmental stress throughout the breeding season, and assessed the survival, specific growth rate in the first six months post-hatching and weight at six months of age of their offspring from the successive clutches.

The results of my study provide evidence of age-dependent inter-clutch differences in offspring early life survival. The quality of offspring of non-stressed females, as indicated by their survival rate in the first three months of life, declined with clutch number through the breeding season, with offspring from the first clutches having higher survival. In contrast, stress-exposed females produced offspring with higher survival rates in later clutches. Later in life, offspring of stress-exposed females had lower survival than those of controls. I also observed an inter-clutch pattern in growth rate, with offspring produced later in the season having a faster initial growth rate but slower growth rate later on, independently of maternal treatment.

These results suggest increased maternal investment by stressed mothers later in the breeding season, manifested by greater initial offspring survival. However, the offspring did not experience the same stressful environment and thus the resulting phenotype may not have been advantageous in the long term, possibly explaining the increased mortality later in life. Faster growth rate in late-produced offspring is likely to be a strategy to increase in size prior to winter, but a physiological cost of accelerated growth may have led to a decrease in growth rate later on.

Introduction

Maternal effects, broadly defined as any non-genetic maternal contribution to offspring phenotype, are thought to be one of the major driving forces behind phenotypic variation seen in the animal kingdom (Mousseau and Fox, 1998, Wolf and Wade, 2009). Mothers have the potential to alter offspring pre- and post-natal developmental trajectories independent from any genetic contribution, resulting in changes to a variety of morphological and physiological traits and variation in offspring growth and survival (Bernardo, 1996). Whilst it is widely accepted that many of these non-genetic differences in offspring phenotypic traits arise through differential allocation of maternal resources (Green, 2008, Sheriff and Love, 2013a), there are many maternal factors that may contribute to offspring phenotypic responses. For example, maternal age (Berkeley *et al.*, 2004, Arnold *et al.*, 2018), size (Campbell and Slade, 1995, Raventos and Planes, 2008) and immune status (Martyka *et al.*, 2018), choice of nest site (Mitchell *et al.*, 2015) and maternal rearing environment (Van Leeuwen *et al.*, 2016, Wang *et al.*, 2017a) have all been shown to produce variation in the growth and survival rates of offspring.

Amongst the most important sources of offspring phenotypic plasticity are hormonally-mediated maternal effects. Females, particularly in oviparous species, have the ability to regulate the immediate developmental environment of their offspring by depositing hormones in eggs/embryos (Dufty *et al.*, 2002, Groothuis *et al.*, 2005, Ruuskanen and Hsu, 2018). The most commonly documented examples of maternally-derived hormones that are associated with offspring phenotypic change are the steroid hormones, with androgens being extensively studied in this context and shown to have an impact on offspring survival (Sockman and Schwabl, 2000, Rutkowska and Cichon, 2006) and growth (Eising *et al.*, 2001, Uller *et al.*, 2007). In addition, in recent years glucocorticoid (GC) hormones have become a focus of research on hormone-mediated maternal effects due to their far-ranging consequences for pre- and post-natal developmental trajectories of the offspring (Schreck *et al.*, 2001, Dufty *et al.*, 2002).

When exposed to stressful conditions, animals mount a generalised stress response; this is mediated by the activation of the hypothalamic-pituitary-adrenal (HPA) axis (or hypothalamic-pituitary-interrenal axis, HPI, in fishes) and

results in the secretion of GCs (Mommsen *et al.*, 1999, Romero, 2004). If levels of maternal GCs are high in the period leading up to gamete development, or during early embryonic development, it often results in elevated GC levels in eggs/embryos (Stratholt *et al.*, 1997, Love *et al.*, 2005b, Meise *et al.*, 2016, Ensminger *et al.*, 2018). This elevation has been shown to alter the post-natal growth and survival of offspring, but there is no clear consensus about the direction of this effect. For example, a positive correlation between elevated maternal GCs and offspring growth was observed in American red squirrels (Dantzer *et al.*, 2013), aspic vipers (Dupoue *et al.*, 2016), great tits (Coslovsky and Richner, 2011) and zebra finches (Khan *et al.*, 2016b), but the opposite trend was observed in Japanese quail (Hayward and Wingfield, 2004) and southern fiddler rays (Guida *et al.*, 2017). The relationship between maternal stress and offspring growth may be further confounded by factors such as offspring sex (Uller *et al.*, 2009) and the quality of the natal habitat (Tilgar *et al.*, 2016). There is also conflicting evidence in terms of offspring survival, with maternal stress having variously been shown to have a positive effect (Weber *et al.*, 2018), negative effect (Bian *et al.*, 2011) or no effect (Polich *et al.*, 2018) on the number of surviving offspring.

The effects of maternal stress and elevated egg GCs (cortisol) on offspring phenotype are considerably less well studied in fish, but the available evidence is similarly inconclusive about the direction of these effects. Offspring growth is often unrelated to pre-hatching cortisol levels (Li *et al.*, 2010, Giesing *et al.*, 2011, Redfern *et al.*, 2017), but it has also been shown to be reduced following maternal stress treatment (Eriksen *et al.*, 2006, Eriksen *et al.*, 2015). Post-hatching survival showed a downward trend when females were implanted with cortisol prior to spawning (Eriksen *et al.*, 2006), but it was increased following immersion of eggs in exogenous cortisol solution (Gagliano and McCormick, 2009, Capelle *et al.*, 2016).

The lack of consensus on the effect of maternal stress on offspring growth and survival may be related to an ongoing debate about the adaptive potential of maternal effects. Maternal hormones (e.g. glucocorticoids) provide a link between the maternal and predicted offspring environment, and as such they are often regarded as a tool for adaptive maternal programming, which is predicted to result in increased fitness (Mousseau and Fox, 1998, Marshall and Uller, 2007, Love *et al.*, 2013). In this adaptive maternal effects scenario,

females predict the future environment that will be faced by their offspring and actively allocate resources (e.g. GCs) to their eggs in order to shape the phenotype of their offspring (Schreck *et al.*, 2001, Sheriff and Love, 2013a). Alternatively, maternal stress effects may be simply a consequence of living in a stressful environment, where an elevation of maternal GCs is reflected in *in ovo* GC levels due to the passive transfer of these hormones from mother to egg. In this scenario, maternal stress exposure may have deleterious effects on fitness by reducing offspring growth and survival (Heath and Blouw, 1998, Sheriff and Love, 2013a). The timescale of maternal exposure to stress is also of relevance, since the traditional view is that acute stressors initiate an adaptive physiological response for coping with short-term adversity (Mommsen *et al.*, 1999, Romero, 2004), whilst chronic stress exposure has a negative effect on physiological processes and fitness (McEwen, 1998, Sapolsky *et al.*, 2000).

The adaptive potential of maternal stress depends on many factors, including species (Schreck *et al.*, 2001), the interplay between genetic and non-genetic maternal factors (Shama *et al.*, 2014, Pick *et al.*, 2016) and mother-offspring environmental matching (Hoyle and Ezard, 2012, Shama, 2015, Sheriff *et al.*, 2018). The latter may be particularly important in terms of chronic maternal stress if the mother actively adjusts offspring phenotype to better cope with the prevailing environmental conditions (Thayer *et al.*, 2018). The literature to date is heavily biased in terms of taxonomic coverage: a large proportion of the current evidence of maternal stress effects comes from studies on birds, and information on fish comes mostly from salmonids, which may not be typical of fish in general due to their specific ecology and complex life cycles (Quinn, 2005, Jonsson and Jonsson, 2011). In addition, studies of the effects of chronic maternal stress in fish have often used cortisol implants as a proxy of protracted chronic stress. This method suffers from serious limitations, which may include uncertainty as to the rate of cortisol release over time (Crossin *et al.*, 2016), heightened sensitivity to low levels of glucocorticoid hormones following constant long-term exposure (Dufty *et al.*, 2002) and side effects of the implantation process (Hoogenboom *et al.*, 2011). Exposure of ova to exogenous cortisol is also used to mimic elevated maternal GCs. However, considering that stressed mothers can program the phenotype of their offspring through factors such as nutrients, androgens etc. (Gil *et al.*, 2004, Henriksen *et al.*, 2011),

manipulation of glucocorticoids alone may not reveal the true phenotypic effect of chronic maternal stress.

It is therefore unclear what consequences maternal exposure to a combination of environmental and husbandry stressors, likely to be encountered by fish, has for the phenotype of the offspring in terms of their post-hatching growth and survival. Moreover, very little research has been conducted to assess the impact of chronic maternal stress on growth and survival of offspring from successive clutches produced over the course of a breeding season if the adverse environmental conditions persist. In this study, I addressed these questions by exploring the effects of simulated chronic maternal stress on offspring post-hatching growth and survival, using a small, non-salmonid fish, the three-spined stickleback. I exposed stickleback females to a period of unpredictable chronic stress and measured the phenotypic response of their offspring in terms of their growth and survival in the first 6 months post-hatching. Applying the stressors throughout the breeding season allowed me to investigate potential maternal adjustments to the phenotype of the offspring from successive clutches. I predicted that stress-exposed females would adjust larval developmental trajectories to produce offspring that are better able to cope with the stressful environment by having higher growth and survival rates. Sticklebacks mostly only reproduce in a single breeding season, during which they spawn multiple times. I therefore expected faster growth, and possibly higher survival rates, in the larvae/juveniles from the clutches produced later in the breeding season: such inter-clutch differences could arise from an alteration of maternal relative investment and/or adjustments to offspring phenotype, as a strategy of females to maximise their fitness towards the end of their reproductive lifespan and increase the overwinter survival potential of their offspring.

Methods and materials

Study system and fry husbandry

The maternal population of three-spined sticklebacks was wild-caught in the River Endrick, western Scotland, in the winter preceding the breeding season and then kept in laboratory conditions. A proportion of the females (Experimental group) were exposed to an unpredictable chronic stress protocol (UCSP), with the remaining females acting as controls. Chapter 2 provides details on fish husbandry, experimental set up and the UCSP. Once the females became gravid, their eggs were stripped under anaesthesia and fertilised *in vitro* with sperm from randomly selected, non-stressed males. To investigate inter-clutch differences in offspring phenotype, up to three clutches produced by each female were crossed with different males across the course of the breeding season. Mean initial size of fry from each clutch was determined on the day of hatching. Details of the *in vitro* procedure and fry size at hatching measurement are provided in Chapter 3.

The hatched fry were retained in their original hatch tanks (10 L clear plastic tanks, 17x19x32 cm). The tap in each tank was set to a slow drip to ensure a constant influx of filtered and UV-sterilised water; the outflow was fitted with fine mesh to prevent fry from being flushed out. Each tank was fitted with an air stone for aeration. From day 1 post-hatching (ph), fry were fed twice daily with a small amount of <100 microns commercial fry pellet (ZM-000, ZM Systems). From day 5 ph, the pellet was supplemented with live *Artemia* nauplii, provided once daily. At 5 weeks ph, fry pellet was substituted with commercial granular food (ZM Small Granular, ZM Systems), ground up with a pestle to reduce particle size. From day 90 ph, supplementary feeding with *Artemia* was reduced to twice weekly and an artificial plant was added to each tank for shelter. The tanks were siphoned on alternate days to remove excess food.

Due to the small size of the tanks, it was necessary part-way through fry development to reduce the size of each family group to contain no more than 15 fry. This was done on day 60 ph by culling randomly-selected excess fry with an overdose of an anaesthetic (50mg/L benzocaine solution). On day 60 ph prior to culling the range of family group sizes was 1-34 fry in the Control group and 1-55 fry (1-36 fry if two clutches produced by a single female, both containing 55 fry, are excluded) in the Experimental group. 29 out of 53 Control families and 22

out of 50 Experimental families did not require size reduction. In the remaining families, an average of 10.7 (±1.67; Control) and 10.9 (±1.59; Experimental) fry were culled.

Assessing fry survival and growth

To determine the proportion of surviving fry and juveniles at various points of their development, they were counted on days 14, 30, 60, 90 and 180 post-hatching (dph). Each tank was illuminated from the bottom on a lightbox and the number of fry was determined visually.

At 30, 60 and 90 dph, fry were measured to determine their growth rate. Depending on the number of surviving fry, up to 10 individuals were transferred into a 90mm Petri dish (or a borosilicate crystallising dish at 90 dph) filled with tank water and placed on a lightbox. The excess water was removed, so that all fry were at the same depth, and the fry photographed with a piece of millimetre paper for scale (Figure 4.1).

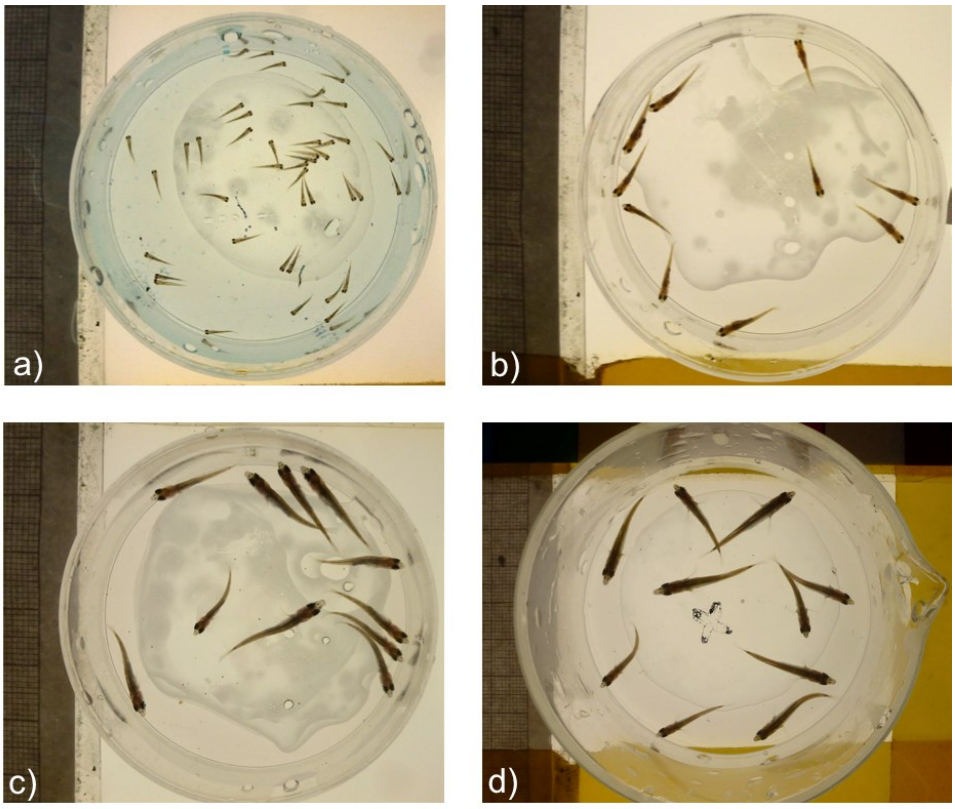

Figure 4.1 Examples of photographs used to measure stickleback fry length at different time points post-hatching: a) Day 1 ph, b) Day 30 ph, c) Day 60 ph, d) Day 90 ph.

The mean standard length (i.e. excluding caudal fin) of fry from each family group (up to 10 fry per family) was determined using ImageJ image processing software (Rasband, 2016). The millimetre paper included in the photograph was

used to set up scale for each photograph separately, and the length was measured by drawing a straight line from the tip of the snout to the base of the caudal fin. To determine an average size at day 180 ph (by which time fish were too large for the same protocol of measuring in a dish), all individuals in the family group were lightly anaesthetised in benzocaine solution (25mg/L). Each fish was blotted dry and its wet mass was recorded to 0.001g. The standard length (to 0.1 mm) was measured using Vernier callipers.

Data analysis

Statistical analyses of fry survival and growth were restricted to the females that produced two or three clutches, to allow for comparison between the successive clutches produced by a female across the breeding season.

The proportion of the family that was alive at each of the analysed time points post hatching (14, 30, 60, 90 and 180 dph) was calculated by dividing the number of surviving fry in a given family by the number of fry that hatched in that family. After excluding observations with missing data on any of the variables, the final sample size for survival analysis included 30 observations of Clutch 1 (Experimental n=15, Control n=15), 30 observations of Clutch 2 (Experimental n=14, Control n=16) and 23 observations of Clutch 3 (Experimental n= 11, Control n=12). Five separate Generalised Linear Mixed Models (GLMM) with a binomial distribution (one for each of the analysed time points post hatching) were used to analyse potential differences in the survival of fry produced by mothers from the two treatment groups. The response variable in these models was the proportion of fry in a family that were alive at each of the time points. The fixed factors included in the initial models were treatment group (categorical variable), clutch number (categorical variable, being either the 1st, 2nd or 3rd clutch of a female), clutch size (covariate; with the exception of survival at 90 dph and 180 dph, where the clutch size was not relevant due to the earlier reduction of family size) and interaction term treatment group*clutch number. To control for the relative influence of the number of fry in a family on the response variable, I used a "weights" argument in the model fitting process, where "weights" is the number of fry in the family (surviving + dead) used to generate each proportion (i.e. the number of surviving fry in the family). Maternal ID was included as a random factor here and in later analyses that used

multiple measurements (i.e. clutches) from the same female. For all models, non-significant terms were removed by backwards selection.

Specific Growth Rate (SGR) at each of the analysed time periods post hatching (1-30 days, 30-60 days, 60-90 days, 90-180 days ph), expressed as % length gain per day, was calculated according to the following formula:

$SGR = [(Log_n \text{ Final Length} - Log_n \text{ Initial Length})/\text{no. of days elapsed}] * 100$

After excluding observations with missing data on any of the variables, the final sample sizes for SGR analysis varied slightly between the time periods. Table 4.1 provides detailed information on the sample sizes used.

Table 4.1 Sample sizes (number of clutches) used for the analysis of Specific Growth Rate (SGR) of fry and juvenile three-spined sticklebacks from different treatment groups (Experimental and Control) and clutches, at different time periods (defined as days post hatching, dph).

Clutch no.	1-30 dph		30-60 dph		60-90 dph		90-180 dph	
	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control
1	7	11	6	11	6	11	7	11
2	4	10	4	11	5	10	5	10
3	7	11	7	10	7	10	7	10

Four separate Linear Mixed Models (LMM), one for each of the analysed time periods, were used to analyse potential differences in SGR of fry produced by mothers from the two treatment groups. The fixed factors included in the initial models were treatment group (categorical variable), clutch number (categorical variable, being either the 1st, 2nd or 3rd clutch of a female), group size at the beginning of each time period (covariate), mean initial length of fry (covariate; length at the start of each time interval, with the exception of 1-30 dph, for which the size at hatching was used), and interaction term treatment group*clutch number.

An LMM was used to analyse potential differences in mass at 180 days ph of juveniles produced by mothers from the two treatment groups. The fixed factors included in the initial models were treatment group (categorical variable), clutch number (categorical variable, being either the 1st, 2nd or 3rd clutch of a female), group size at the time of measurement (covariate), and interaction term treatment group*clutch number. Because individual mass of each fish in a family was known, after excluding observations with missing data on any of the

variables the final sample size included 317 observations of Clutch 1 (Experimental n=166, Control n=151, being the offspring of 15 Experimental and 15 Control mothers), 243 observations of Clutch 2 (Experimental n=117, Control n=126, 12 and 13 mothers respectively) and 242 observations of Clutch 3 (Experimental n= 117, Control n=125, 11 and 13 mothers respectively).

All statistical analyses were performed in R statistical software (version 3.5.2, R Development Core Team), with GLMMs and LMMs fitted using the "lme4" package in R (Bates *et al.*, 2015). The degrees of freedom were estimated by Satterthwaite approximation (for normally distributed data) and Laplace approximation (for data with binomial distribution). P values were obtained from t-statistics or z-statistic using the "lmerTest" package, with an alpha level of 0.05 (Kuznetsova *et al.*, 2017).

Results

Fry survival

Exposure of a female to a period of chronic stress altered the patterns of survival of her offspring from successive clutches produced across the breeding season (Figure 4.1, Table 4.2). At 14 days post hatching (dph), the difference was apparent with regards to Clutch 2, which in the Experimental group had higher survival than Clutch 1 and Clutch 3, whereas in the Control group Clutch 2 had the lowest survival of the three clutches. The interaction between treatment group and clutch number was also significant at 30 dph and 60 dph: whilst in the Experimental group fry from a female's first clutch had lower survival than those from later clutches, in the Control group the proportion of surviving fry was significantly higher in the first clutches. The direction of this effect changed at 90 dph, with clutches produced later in the breeding season (Clutch 3) having significantly lower offspring survival in the Experimental group, but significantly higher offspring survival in the Control group. Any differences between treatment groups and clutches had diminished by day 180 ph. Clutch size had a small but statistically significant positive effect on fry survival at 30 dph only (Figure 4.2, Table 4.2).

Specific Growth Rate

SGR did not differ between the offspring of the stress-exposed and non-exposed females at any of the time periods analysed. There was no significant difference in SGR between the successive clutches produced by a female across the breeding season until 60 dph. However, fry from Clutch 3 grew at a faster rate than fry from Clutch 1 or Clutch 2 between 60 and 90 dph (Figure 4.3, Table 4.3), and at a slower rate than the first two clutches between 90 and 180 dph (Figure 4.4, Table 4.3), regardless of the maternal treatment. The size of the family group had a significant effect on SGR up to 60 dph, with fry from larger groups having lower growth rates. Growth rate was also negatively correlated with mean fry length at the beginning of each time period (Figure 4.4, Table 4.3).

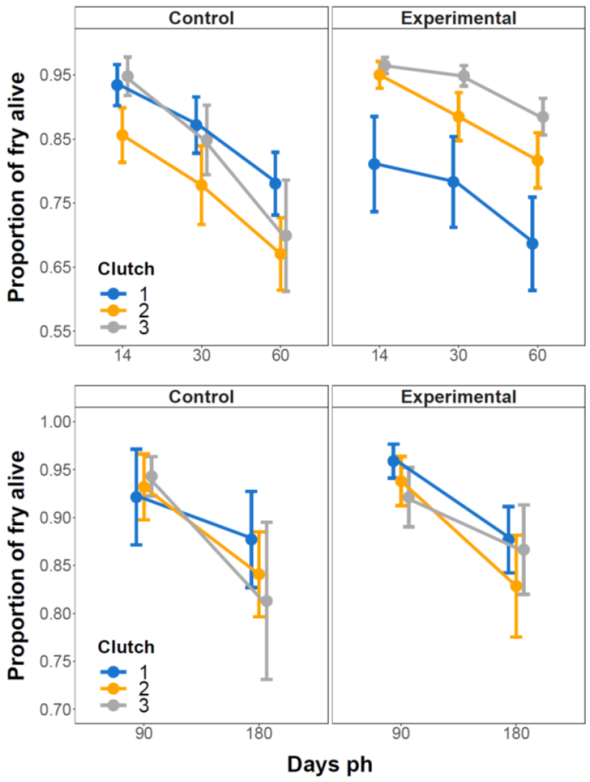

Figure 4.1 Differences in three-spined stickleback fry survival (as proportion of fry alive at each time point) in three successive clutches produced across the breeding season by females from Control and Experimental treatment groups. The sample sizes were 30 observations of Clutch 1(Exp. n=15, Ctrl n=15), 30 observations of Clutch 2 (Exp. n=14, Ctrl n=16) and 23 observations of Clutch 3 (Exp. n=11, Ctrl n=12). The top panels illustrate fry survival at days 14, 30 and 60 post hatching, prior to the reduction of the family size to ≤15 fry. The bottom panels illustrate fry survival at days 90 and 180 ph, following the reduction in family size. The error bars represent Standard Error of the Mean (s.e).

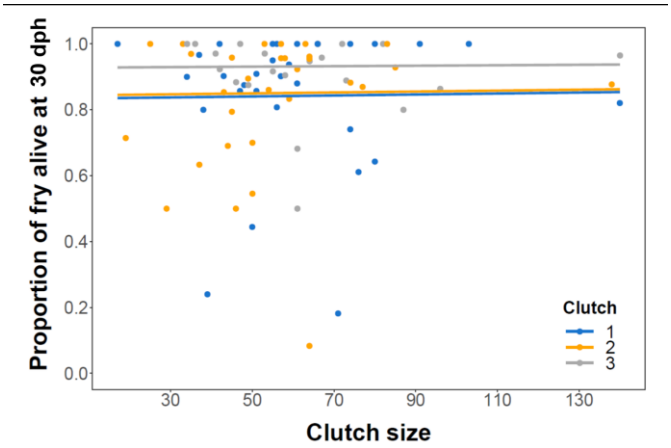

Figure 4.2 The relationship between fry survival at day 30 post hatching (as proportion of fry alive) and clutch size in three successive clutches produced across the breeding season by three-spined stickleback females. The sample size was 30 observations of Clutch 1, 30 observations of Clutch 2 and 23 observations of Clutch 3. Due to the lack of difference in offspring survival between the treatment groups, the figure shows combined data for the Control and Experimental fish. The regression lines were predicted from a model including treatment group, clutch number (and their interaction) and clutch size as fixed effects.

Table 4.2 Summary of Generalised Linear Mixed Models (GLMM) used to analyse the differences in fry survival in three successive clutches produced by Control and Experimental female three-spined sticklebacks during the breeding season, at five different time points post hatching (measured as days post hatching, dph). The reference Treatment was the Control group, and the reference clutch was the first clutch in the season. Female ID was included in the models as a random factor. Clutch 1 n=30 (Exp. n=15, Ctrl n=15), Clutch 2 n=30 (Exp. n=14, Ctrl n=16), Clutch 3 n=23 (Exp. n=11, Ctrl n=12). Treatment group, Clutch and all significant terms (indicated with p-values in underlined and bold font) were retained in the final model.

Model terms	**Effect on fry survival at:**				
	14 dph	**30 dph**	**60 dph**	**90 dph**	**180 dph**
Treatment group	-0.574 ± 0.595, z=-0.966, p=0.334	-0.313 ± 0.435, z=-0.720, p=0.471	-0.091 ± 0.282, z=-0.322, p=0.748	0.645 ± 0.603, z=1.070, p=0.285	-0.200 ± 0.313, z=-0.642, p=0.521
Clutch 2	-0.608 ± 0.360, z=-1.687, p=0.092	-0.473 ± 0.285, z=-1.659, p=0.097	-0.143 ± 0.207, z=-0.693, p=0.488	0.519 ± 0.470, z=1.105, p=0.269	-0.204 ± 0.230, z=-0.885, p=0.376
Clutch 3	1.173 ± 0.488, z=2.406, **p=0.016**	0.540 ± 0.326, z=1.656, p=0.098	0.241 ± 0.230, z=1.047, p=0.295	0.726 ± 0.475, z=1.530, p=0.126	0.058 ± 0.252, z=0.230, p=0.818
Treatment group*Clutch 2	2.199 ± 0.511, z=4.305, **p<0.001**	1.608 ± 0.380, z=4.234, **p<0.001**	0.959 ± 0.279, z=3.431, **p<0.001**	-1.039 ± 0.677, z=-1.536, p=0.124	-0.572 ± 0.465, z=-1.230, p=0.219
Treatment group*Clutch 3	0.676 ± 0.624, z=1.082, p=0.279	1.147 ± 0.461, z=2.490, **p=0.013**	1.093 ± 0.321, z=3.407, **p<0.001**	-1.452 ± 0.694, z=-2.094, **p=0.036**	-0.366 ± 0.506, z=-0.724, p=0.469
Clutch size	0.019 ± 0.011, z=1.827, p=0.068	0.019 ± 0.007, z=2.481, **p=0.013**	0.007 ± 0.005, z=1.362, p=0.173	NA	NA

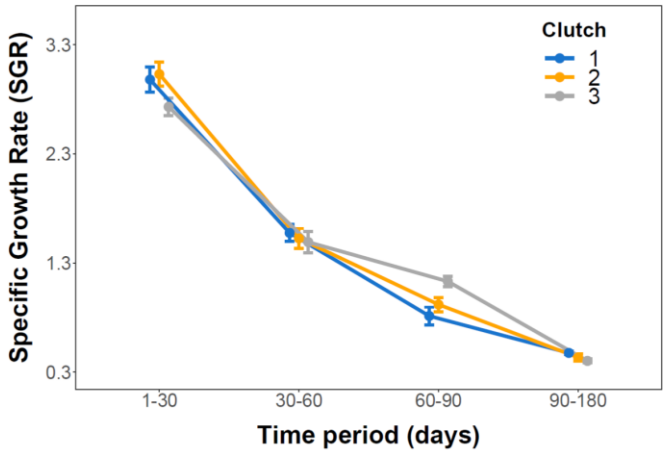

Figure 4.3 Specific Growth Rate (SGR; measured as % length increase per day) at different time periods post hatching of three-spined stickleback fry from three successive clutches produced across the breeding season. Due to the lack of difference in offspring survival between the treatment groups, the figure shows combined data for the Control and Experimental fish. The sample sizes are provided in Table 4.1. The error bars represent Standard Error of the Mean (s.e).

Table 4.3 Summary of Linear Mixed Models (LMM) used to analyse the differences in Specific Growth Rate (SGR, as % length increase per day) in fry from the three successive clutches produced by Control and Experimental female three-spined sticklebacks during the breeding season, at four different time periods post hatching (as days post hatching, dph). The reference Treatment was the Control group, and the reference clutch was the first clutch in the season. Female ID was included in the models as random factor. The sample sizes are provided in Table 4.1. Treatment group, Clutch and all significant terms (indicated with p-values in underlined and bold font) were retained in the final models.

Model terms	**Effect on SGR:**			
	1-30 dph	**30-60 dph**	**60-90 dph**	**90-180 dph**
Treatment group	0.106 ± 0.117, $t_{1,29.97}$=0.909, p=0.371	0.081 ± 0.091, $t_{1,22.28}$=0.893, p=0.381	-0.047 ± 0.086, $t_{1,26.33}$=-0.550, p=0.587	0.002 ± 0.029, $t_{1,25.51}$=0.085, p=0.933
Clutch 2	0.089 ± 0.128, $t_{1,35.71}$=0.691, p=0.494	-0.060 ± 0.076, $t_{1,22.28}$=-0.795, p=0.435	0.103 ± 0.085, $t_{1,32.86}$=1.220, p=0.231	-0.027 ± 0.025, $t_{1,29.48}$=-1.098, p=0.281
Clutch 3	-0.094 ± 0.127, $t_{1,37.68}$=-0.741, p=0.463	-0.085 ± 0.074, $t_{1,24.04}$=-1.155, p=0.463	0.306 ± 0.081, $t_{1,31.54}$=3.774, **p=<0.001**	-0.060 ± 0.024, $t_{1,29.51}$=-2.486, **p=0.019**
Treatment group*Clutch 2	-0.302 ± 0.275, $t_{1,38.80}$=-1.099, p=0.279	0.136 ± 0.173, $t_{1,34.84}$=0.789, p=0.260	0.268 ± 0.187, $t_{1,36.95}$=1.434, p=0.160	-0.007 ± 0.055, $t_{1,33.28}$=-0.134, p=0.894
Treatment group*Clutch 3	-0.335 ± 0.245, $t_{1,35.08}$=-1.369, p=0.180	0.291 ± 0.154, $t_{1,29.73}$=1.890, p=0.069	0.116 ± 0.175, $t_{1,34.94}$=0.665, p=0.511	0.034 ± 0.050, $t_{1,29.82}$=0.668, p=0.509
Group size	-0.010 ± 0.004, $t_{1,39.90}$=-2.265, **p=0.029**	-0.020 ± 0.004, $t_{1,37.86}$=-5.342, **p<0.001**	-0.002 ± 0.011, $t_{1,39.99}$=-0.216, p=0.830	-0.002 ± 0.003, $t_{1,38.86}$=-0.721, p=0.475
Initial length	-4.489 ± 1.483, $t_{1,40.74}$=-3.027, **p=0.004**	-1.231 ± 0.245, $t_{1,29.81}$=-5.020, **p<0.001**	-0.300 ± 0.136, $t_{1,42.59}$=-2.209, **p=0.033**	-0.080 ± 0.028, $t_{1,42.38}$=-2.810, **p=0.007**

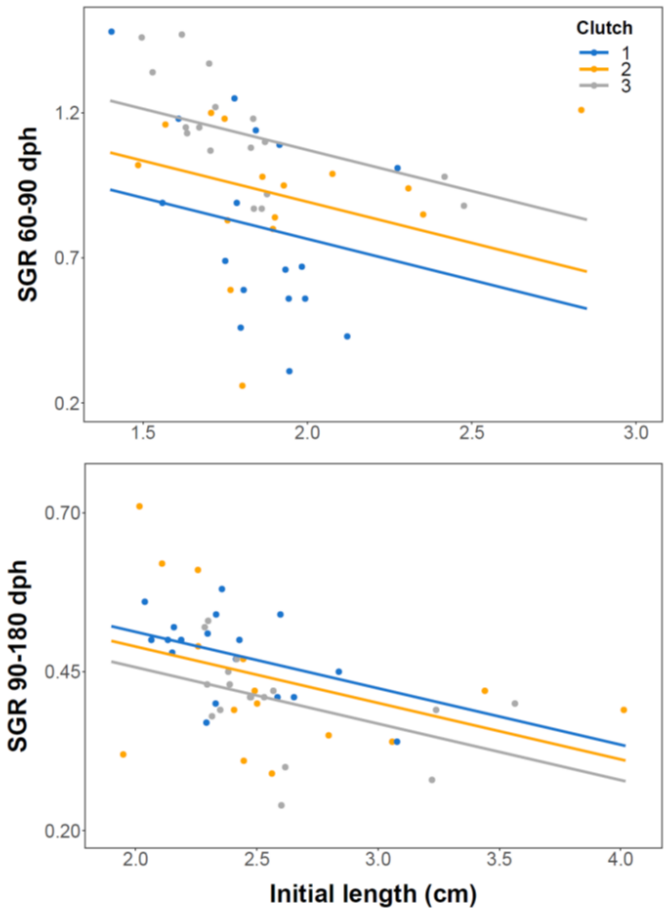

Figure 4.4 The relationship between Specific Growth Rate (SGR, as % length increase per day) at 60-90 days post hatching (dph; top panel) and 90-180 dph (bottom panel), and fry length (in cm) at the beginning of a given time period, in three successive clutches produced across the breeding season by three-spined stickleback females. Due to the lack of difference in offspring survival between the treatment groups, the figure shows combined data for the Control and Experimental fish. The sample size is provided in Table 4.1. The regression lines were predicted from a model including treatment group and clutch number (and their interaction) as fixed effects.

Mass at 180 dph

At 180 dph, offspring of stress-exposed mothers did not differ in mass from those of non-exposed mothers (Figure 4.5). There was a small inter-clutch difference in mass, with offspring from clutches produced later in the breeding season (Clutch 2 and 3) being lighter (Table 4.4). This difference, however, was statistically significant only in fry from Clutch 2, which were lighter than those from Clutch 1. There was also a strong negative correlation between the mass of fry at 180 dph and the size of the family group (Table 4.4).

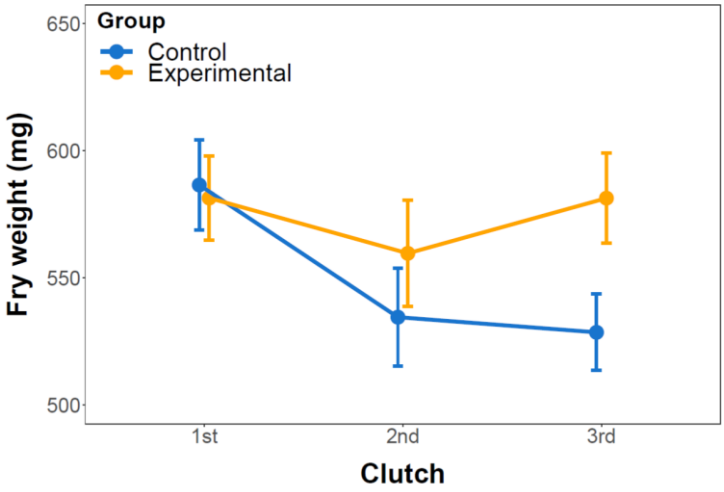

Figure 4.5 Mass (in mg) at 180 days post hatching of three-spined stickleback fry from three successive clutches produced across the breeding season. The sample size included 317 observations of Clutch 1 (Experimental n=166, Control n=151, offspring of 15 Experimental and 15 Control mothers), 243 observations of Clutch 2 (Experimental n=117, Control n=26, 12 and 13 mothers respectively) and 242 observations of Clutch 3 (Experimental n= 117, Control n=125, 11 and 13 mothers respectively). The error bars represent Standard Error of the Mean (s.e).

Table 4.4 Summary of Linear Mixed Model (LMM) used to analyse the differences in mass (in mg) in fry from the three successive clutches produced by Control and Experimental female three-spined sticklebacks during the breeding season, at 180 days post hatching (dph). The reference Treatment was the Control group, and the reference clutch was the first clutch in the season. Female ID was included in the models as random factor. The sample size included 317 observations of Clutch 1 (Experimental n=166, Control n=151, offspring of 15 Experimental and 15 Control mothers), 243 observations of Clutch 2 (Experimental n=117, Control n=26, 12 and 13 mothers respectively) and 242 observations of Clutch 3 (Experimental n= 117, Control n=125, 11 and 13 mothers respectively). Treatment group, Clutch and all significant terms (indicated with p-values in underlined and bold font) were retained in the final model.

	Effect on fry mass at 180 dph			
Model terms	**Est.**	**s.e.**	**t value**	**p value**
Treatment group	15.901	25.747	0.618	0.542
Clutch 2	-32.787	16.532	-1.983	**0.048**
Clutch 3	-25.174	16.618	-1.515	0.130
Treatment group*Clutch 2	-3.485	33.001	-0.106	0.916
Treatment group*Clutch 3	53.526	33.088	1.618	0.106
Group size	-25.705	2.323	-11.065	**<0.001**

Discussion

The effect of chronic maternal stress on offspring survival

The results of this study provide an evidence of inter-clutch differences in offspring early life survival following the exposure of female sticklebacks to chronic stress in the period leading up to egg production and spawning. When maternal treatment on its own was taken into account, there was no significant difference in survival between offspring of stress-exposed and non-exposed females, neither immediately after hatching nor later on. This is congruent with the evidence from sockeye salmon (Sopinka *et al.*, 2014) and common lizards (Meylan and Clobert, 2005), where maternal stress treatment (repeated acute stressor exposure and corticosterone injections, respectively) did not have an impact on offspring survival. There is however no clear consensus as to this effect, with other studies on a range of species showing increased mortality following maternal stress exposure (Eriksen *et al.*, 2006, Eriksen *et al.*, 2007, Bian *et al.*, 2011). Conversely, maternal stress (or exposure to exogenous GCs) has also been shown to confer a survival advantage for offspring (Gagliano and McCormick, 2009, Capelle *et al.*, 2016, Wang *et al.*, 2017a).

Offspring phenotypic response to maternal stress may depend on many additional factors, such as maternal early life conditions (Burton *et al.*, 2013), maternal age (Berkeley *et al.*, 2004) and size (Raventos and Planes, 2008), the nature of the stressor (Jafari *et al.*, 2017) and offspring sex (Rutkowska and Cichon, 2006). In addition, where exogenous GCs are used to mimic maternal stress, the effects may be highly dose-dependent (Midwood *et al.*, 2014, Gagliano and McCormick, 2009). Acting in various combinations, these factors may contribute to the variability of the observed effects.

Despite not having a direct effect on offspring survival, exposure of female sticklebacks to chronic stress significantly affected the relationship between offspring post-hatching survival, maternal treatment and clutch order. In animals reproducing more than once in a breeding season, such as serially spawning fish, mothers have to partition their energy resources between successive clutches/litters, which may result in inter-clutch differences in clutch characteristics (as considered in Chapter 3 of this thesis), offspring survival, growth etc. Such seasonal maternal adjustments have previously been documented e.g. in ambon damselfish, where the size of fry declined but the

size of yolk sacs increased over the breeding season, with the potential to increase the probability of survival of later clutches (Maddams and McCormick, 2012). In addition, maternal investment strategy may be influenced by environmental conditions. If the environmental conditions are stable, females can reliably anticipate the environment that their offspring will encounter upon hatching/birth and adjust the phenotype of the offspring accordingly (Proulx and Teotonio, 2017). However, in an unpredictable environment it may be more beneficial for females to adopt a bet-hedging strategy, i.e. produce offspring that vary in characteristics across the breeding season, with the prospect that at least some of these characteristics will provide a survival advantage (Crean and Marshall, 2009).

In the present study, the quality of offspring (indicated by their survival rate in the first three months) of Control females declined across the breeding season, whilst females exposed to a period of chronic stress produced higher quality offspring later in the season. However, later in life the offspring of stress-exposed females had lower survival than those of Controls. It is unclear how this observation fits in with either the randomising (i.e. producing offspring with varying characteristics) or deterministic (i.e. adjusting offspring phenotype to the anticipated offspring environment) maternal effect strategy. The bet-hedging model of Crean & Marshall (2009) would predict that exposure to a stressful and unpredictable environment (such as that produced by simulated environmental stress in this experiment) would favour increased variation in offspring phenotype, but long-term stress exposure may provide females with reliable information on the anticipated offspring environment, thus favouring adjustment of their phenotype to provide a survival advantage (Proulx and Teotonio, 2017). Since the offspring did not experience the same stressful environment as their mothers, the resulting phenotype may not have been the most advantageous in the long term, leading to an increased mortality after the first three months.

A possible explanation of the observed results stems from the study of Mitchell *et al*. (2018) on brown anole lizards, where females produced higher quality offspring later in the breeding season if the environmental conditions deteriorated over the course of the season. The results of behavioural tests performed on female sticklebacks during the breeding season provided no clear evidence of habituation to the stress protocol (Chapter 2 of this thesis). It is

therefore possible that accumulation of the stress effects in females led to a perceived deterioration in the quality of the environment, resulting in increased maternal investment later in the breeding season.

The survival of stickleback fry to 30 days post hatching was also influenced by the clutch size from which they hatched, with fry from larger clutches having a lower mortality. In this laboratory setting, where each family occupied a separate tank, larger clutches resulted in higher density of the family groups. It has been previously demonstrated, e.g. in fish (Forrester, 1995, Johnson, 2008) and birds (Mallord *et al.*, 2007, Styrsky *et al.*, 2005, Payo-Payo *et al.*, 2016), that a higher density of individuals results in lower survival, both early in life and in adulthood, which is at odds with my findings. There is some limited evidence of female attempts to increase their reproductive success by increasing both the number and the quality (in terms of survival) of their offspring (Lepage *et al.*, 1998, Sinervo and McAdam, 2008). However, in my study the correlation between the clutch size and survival rate should be treated with caution, as it may be simply an indicator of female quality, with better quality females producing both larger clutches and offspring with higher survival potential. An experimental approach involving clutch size manipulation would be required to disentangle this relationship.

Inter-clutch differences in growth rate

The growth rate of the young sticklebacks was the highest in the first 30 days post hatching and slowed down later on. This is consistent with the theory that as animals grow they must allocate more resources into maintenance of the existing soma, at the cost of investing in further growth (Dmitriew, 2011, Sibly *et al.*, 2015, Van Buskirk *et al.*, 2017). This would also explain the negative relationship between the initial length and growth rate at each of the time periods observed in the present study. No correlation was observed between maternal stress exposure and offspring growth rate. This indicates that adaptive maternal programming in terms of offspring growth did not occur following a period of chronic stress, but neither did maternal stress have a deleterious effect in this context. This is consistent with the findings from a range of taxa, including fish (Li *et al.*, 2010, Giesing *et al.*, 2011, Redfern *et al.*, 2017), birds (Rubolini *et al.*, 2005) and reptiles (Cadby *et al.*, 2010).

However, other studies carried out across a number of species and contexts do not provide consistent evidence on the direction of the maternal stress effect on offspring growth. In certain situations, exposure to maternally-derived or exogenous glucocorticoids has resulted in increased growth rate of juveniles (Dantzer *et al.*, 2013, Dupoue *et al.*, 2016, Khan *et al.*, 2016b), but it has also been reported to have a negative effect on growth (Hayward and Wingfield, 2004), which can be sex- and habitat-specific (Uller *et al.*, 2009, Tilgar *et al.*, 2016) and dependent on the nature and intensity of the stressors acting upon females (Kleist *et al.*, 2018). In the present study, behavioural but not hormonal responses were observed following the exposure of females to chronic stress (Chapter 2), and no increase in cortisol was apparent in eggs (Chapter 3), suggesting factors other than glucocorticoid hormones mediated the effects of maternal stress. This hinders the direct comparisons of this study with those where maternal/egg GCs were naturally or artificially elevated.

Regardless of maternal treatment, I observed a significant inter-clutch difference in growth rate, with the fry produced later in the breeding season showing a distinct growth pattern after the first two months post hatching: these fry had higher growth rates than those from the earlier clutches between 60 and 90 days post hatching, but they grew more slowly between 90 and 180 days. There are some examples from studies on birds of faster growth in chicks hatching from eggs later in the laying sequence of a clutch (You *et al.*, 2009) and higher growth rate in chicks produced later in the season (Eising *et al.*, 2001). There is, however, a lack of previous evidence from fish studies that mothers adjust their provisioning of clutches produced later in the season, resulting in higher growth rate. A possible explanation for the higher growth rate observed in sticklebacks from the later clutches is related to the overwinter survival of young fish, which tends to be positively correlated with body size attained by the onset of winter (Haramis *et al.*, 1986, Smith, 2002, Schorr *et al.*, 2009, Shoup and Wahl, 2011, Midwood *et al.*, 2017). Therefore there is an increased pressure on the individuals from the late clutches to grow faster and reach a size that is optimal for survival before winter. Synthesis of proteins required for somatic growth bears a cost in terms of energy and resources (Wieser, 1994, Peterson *et al.*, 1999, Dmitriew, 2011), as well as increased oxidative damage (Mangel and Munch, 2005, Smith *et al.*, 2016). Thus I propose that the subsequent reduction in growth rate observed in fry from clutches produced late

in the season may be related to a delayed physiological cost of the earlier rapid growth.

As with survival rate, growth rate is often density dependent, with higher densities resulting in slower somatic growth (Grant and Imre, 2005, Lobon-Cervia, 2007, Harding *et al.*, 2018). In the present study, the growth rate of stickleback fry was negatively correlated with the size of the family group during the first two months. The size of the groups was experimentally reduced at 60 dph and the fish were fed *ad libitum* and thus not food-limited; it is therefore possible that competition for space and resources was no longer a factor constraining the growth rate later in juvenile life.

Juvenile mass at 180 dph

The analysis of the juvenile stickleback body mass at six months post hatching showed no evidence in support of adaptive maternal programming in response to chronic environmental stress. Body size and body mass-based condition are generally considered to be positively correlated with reproductive success (Salvador *et al.*, 2008, Milenkaya *et al.*, 2015, Roney *et al.*, 2018, but see Barnett *et al.*, 2015, and Wilder *et al.*, 2016, who argue that these two indices may not be related in a straightforward way). Therefore, under a scenario whereby maternal stress elicits an adaptive response, it would be beneficial for mothers exposed to an unpredictable or stressful environment to alter the phenotype of their offspring towards a larger body size and better condition, resulting in increased chances of survival and reproductive success if offspring encounter this unfavourable environment. A positive relationship between maternal stress and offspring body mass has been previously reported in rats (Amugongo and Hlusko, 2014), guinea pigs (Schöpper *et al.*, 2012) and Japanese quail (Guibert *et al.*, 2011). However, the opposite effect is generally reported in fish (Eriksen *et al.*, 2015, Capelle *et al.*, 2016, Guida *et al.*, 2017), and the present study found no effect of maternal stress on body mass, suggesting that the impact of maternal stress on juvenile mass may be species- and context-dependent.

The observed small inter-clutch differences in mass at six months, independent of maternal treatment, do not follow the expectations stemming from the ecology of three-spined sticklebacks. In an annual population of sticklebacks as used in the present experiments (Lee *et al.*, 2012) the trade-off between

maternal somatic maintenance and offspring provisioning may lose its importance towards the end of a female's reproductive lifespan (Poizat *et al.*, 1999). Moreover, offspring produced later in the breeding season have less time to reach a bigger size prior to winter. Combined, these two factors would be expected to result in an increased investment in the late produced offspring to maximise their chances of survival in unpredictable environment, even if this comes at the expense of maternal condition. It is therefore unclear why in this study the later offspring, particularly those produced in the middle of the breeding season, have lower body mass as juveniles.

In summary, in this study I measured the effect of maternal exposure to a period of chronic stress on survival and growth rate of the resulting offspring. To the best of my knowledge, this is the first study to explore the effects of maternal stress exposure on offspring from the successive clutches produced over a single breeding season, or over the reproductive lifespan of females from an annual population of fish. Maternal stress treatment affected the relationship between clutch order and offspring survival in the first six months post hatching, but did not have an effect on offspring growth and their body mass at six months of age. However, this study may be the first one to observe a change in growth pattern between successive clutches, with the direction of this change suggesting an attempt to alter the phenotype of late-produced offspring.

Chapter 5: Offspring behaviour and stress physiology in response to chronic maternal stress

Abstract

Mediated by factors such as maternally derived glucocorticoids, exposure of females to stressful conditions during the period of egg production can have a profound effect on the behaviour of their offspring. As a result, various aspects of the post-natal behavioural phenotype may show altered patterns in terms of the consistency of behavioural patterns and their average level of performance. Maternal stress can also affect the development of the stress axis in the offspring leading to alterations in their physiological stress response. However, the majority of evidence comes from studies utilising acute stressors or exogenous glucocorticoids, and little is known about the effect of chronic maternal stress.

I exposed adult female three-spined sticklebacks to a chronic stress protocol, in which they experienced a range of environmental and husbandry stressors in the period leading up to spawning. I quantified the activity, sheltering and anxiety-like behaviour in the offspring from the successive clutches of these females, and calculated within- (siblings) and between-clutch (half-siblings) similarity scores for these behaviours. High similarity indicated low variability between siblings or half-siblings, and *vice versa*. I also exposed these offspring to an acute stressor and measured their peak cortisol levels.

Exposure of a mother to chronic stress led to diversification in offspring behaviour, indicated by an increased within-family variability in activity levels and use of a shelter. This may be an effect of a bet-hedging strategy adopted by females in a stressful and unpredictable environment, where it would be beneficial to produce offspring differing in behavioural phenotype, and thus increasing the chance that some of these offspring will be able to better cope with the prevailing environmental conditions. Chronic maternal stress had a slight modifying effect on inter-clutch patterns of stress response, with offspring produced later in the breeding season having a tendency to produce less cortisol in response to an acute stressor. This may be a strategy of maximising overwinter survival potential of late-produced offspring if heightened stress reactivity has a negative effect on growth and survival.

Introduction

One of the fundamental mechanisms behind observed patterns of intraspecific variation in animal behaviour is associated with secretion of hormones - chemical messengers acting as a link between an animal and its environment and capable of altering all aspects of behaviour (Hau and Goymann, 2015, Garland *et al.*, 2016). Amongst the most important modulators of behaviour are steroid hormones, which have been shown to affect courtship behaviour (Cornil *et al.*, 2006, Lord *et al.*, 2009), aggression (Soma *et al.*, 2000, Ros *et al.*, 2002, Øverli *et al.*, 2002) and stress responsiveness (Sapolsky *et al.*, 2000, Barton, 2002). The latter is closely associated with glucocorticoid (GC) hormones, released following stimulation of the hypothalamic-pituitary-adrenal (HPA) axis (or hypothalamic-pituitary-interrenal, HPI, axis in fish). Activation of the HPA/HPI axis by an external stimulus (e.g. a stressor) results in a cascade of hormones, with the end products of this cascade being GC hormones, mainly corticosterone and cortisol (Wendelaar Bonga, 1997, Sapolsky *et al.*, 2000, Romero, 2004). These GC hormones have an ability to alter a number of behaviours, including locomotor activity (Øverli *et al.*, 2002, Millot *et al.*, 2014, Krause *et al.*, 2017), feeding behaviour (Leal *et al.*, 2011, Favreau-Peigne *et al.*, 2014), learning (Piato *et al.*, 2011, Raoult *et al.*, 2017), parental care (O'Connor *et al.*, 2009, Vitousek *et al.*, 2014) and anti-predator behaviour (Thaker *et al.*, 2009, Grace *et al.*, 2017).

The effect of stressor exposure on animal behaviour and stress physiology is often directly linked to stress-induced alteration of GC signalling pathways (Kleist *et al.*, 2018), change in responsiveness of the stress axis to subsequent stressors (Rich and Romero, 2005) and differential expression of genes encoding corticosteroid receptors (Lee *et al.*, 2019). Thus, GCs can have a pleiotropic effect on various systems involved in behavioural and physiological stress responses, with the potential for hormones to regulate behaviour and *vice versa* (McGlothlin and Ketterson, 2008, Garland *et al.*, 2016). The link between GC levels and various aspects of behaviour has been demonstrated e.g. in fish (Archard *et al.*, 2012, Raoult *et al.*, 2017) and birds (Tarjuelo *et al.*, 2015), but may not be ubiquitous, as indicated by studies that did not observe this association (Munteanu *et al.*, 2017, Lawrence *et al.*, 2018a).

If a female experiences stressful conditions during the period of egg production or embryo formation, this can potentially have a profound effect on the behaviour and stress physiology of her offspring. One of the best-known mechanisms through which females influence the levels of glucocorticoid hormones in their offspring is the deposition of maternally derived GCs, where GC levels of eggs/embryos reflect those of a mother (Okuliarova *et al.*, 2010, Kleppe *et al.*, 2013, Ensminger *et al.*, 2018). In addition, there is recent evidence of a link between maternal stress exposure and offspring phenotypic change mediated by certain maternal mRNAs deposited in eggs (Colson *et al.*, 2019). Maternally transferred mRNAs and GCs play an important organisational role during neurogenesis and neurodevelopment; changes to their abundance may thus lead to the alteration of cellular pathways in the brain, resulting in phenotypic changes to post-natal behaviour (Weinstock, 2008, Kleppe *et al.*, 2013, Kleist *et al.*, 2018, Best *et al.*, 2017). For example, offspring of stress exposed mothers, as well as animals exposed to exogenous GCs during early development, showed changes in behavioural traits such as activity (Archard *et al.*, 2012, Colson *et al.*, 2015), anti-predatory behaviour (Uller and Olsson, 2006, Robert *et al.*, 2009, Morales *et al.*, 2018), cognitive ability (Eaton *et al.*, 2015, Feng *et al.*, 2015, Munch *et al.*, 2018), fearfulness (Janczak *et al.*, 2006), aggressiveness (Tamilselvan and Sloman, 2017, Burton *et al.*, 2011) and boldness (Sopinka *et al.*, 2015a, Best *et al.*, 2017).

Similarly, pre-natal exposure to elevated levels of glucocorticoids (either through maternal stress exposure or exogenous GCs) may lead to altered expression of genes involved in HPA/HPI axis development, resulting in the variation in baseline GC levels and altered response to stressful stimuli. There is however no clear consensus as to the direction of this effect, particularly in terms of baseline stress hormone levels, which have been shown to be elevated (Bian *et al.*, 2015) or reduced (He *et al.*, 2016, Tilgar *et al.*, 2016, Kleist *et al.*, 2018). Hormonal response to a stressor (measured as a peak GC level following the exposure e.g. to a predator or an acute stressor) of animals pre-natally exposed to either maternal stress or exogenous GCs has also been shown to be suppressed (Jeffrey and Gilmour, 2016, Nesan and Vijayan, 2016, Redfern *et al.*, 2017) or elevated (Cirulli *et al.*, 2009, Soares-Cunha *et al.*, 2018). The magnitude of this response may depend on factors such as sex (Archard *et al.*, 2012) and presence of conspecifics (Mommer and Bell, 2013). In addition, there

may be no difference in baseline GC levels or the magnitude of the hormonal stress response, but the reactivity of the stress axis may nonetheless be altered (Weber *et al.*, 2018).

It is unclear whether maternal stress and its ability to program the behavioural phenotype and stress physiology of offspring has an adaptive or maladaptive effect on their survival and fitness. The adaptive potential of maternal stress in terms of offspring behaviour may be highly dependent on the ecological context (Dufty *et al.*, 2002, Sheriff *et al.*, 2018). The costs and benefits of a behavioural trait produced as a result of maternal stress may be highly dependent on how closely the environment faced by the offspring matches that experienced by the mother. For example, if the mother experiences a high risk of predation, she may alter the behavioural phenotype and stress physiology of her offspring to make them better suited to a high-predation environment (e.g. lower activity levels, improved associative learning, higher risk aversion) and thus provide them with higher survival potential in such an environment (Groothuis *et al.*, 2005, Love *et al.*, 2013, Sheriff and Love, 2013b, Sheriff *et al.*, 2018).

However, if the information provided by a mother via hormones is unreliable (e.g. when the offspring phenotypic change is a side effect of maternal condition rather than an anticipatory maternal effect), the environment is highly changeable and unpredictable, or the stressors encountered by the offspring are novel, the resulting phenotype may be maladaptive and negatively affect fitness (Groothuis *et al.*, 2005, Marshall and Uller, 2007, Hoyle and Ezard, 2012, Sheriff and Love, 2013a, Sheriff and Love, 2013b). In addition, maternal stress is likely to act upon multiple offspring traits simultaneously; therefore, trade-offs between different traits may arise, for example when maternally transferred hormones have a positive effect on offspring growth whilst at the same time negatively affecting the behavioural phenotype (Marshall and Uller, 2007, Uller *et al.*, 2013).

As the above examples indicate, despite the mounting body of evidence, there is still a high degree of uncertainty as to the direction, magnitude and adaptive significance of the effects of maternal stress on offspring behaviour and stress physiology. This is particularly true in fish, where a number of factors may hinder the measurement of this effect as well as the comparison within and between species. Firstly, until relatively recently the measurement of the

maternal and offspring hormonal stress response has been either invasive or destructive due to methodological limitations and the small size of many of the species of fish used in scientific experiments (Ellis *et al.*, 2004, Sebire *et al.*, 2007, Scott and Ellis, 2007). As a result, it has been difficult to disentangle the effect of stress exposure (in mothers) or maternal stress (in offspring) from the effects of stress hormone sampling, and longitudinal studies have usually not been possible.

Secondly, there is a lack of consistency in the methodology used to induce or mimic maternal stress, especially when the aim has been to examine effects of chronic stress. The most widely used methods have included exposure to simulated predation (Mommer and Bell, 2013, Feng *et al.*, 2015), repeating the same acute stressor (Sopinka *et al.*, 2014, Sopinka *et al.*, 2017), cortisol implantation (McConnachie *et al.*, 2012, Eriksen *et al.*, 2015) and injection (Espmark *et al.*, 2008, Redfern *et al.*, 2017), as well as exposing eggs to exogenous cortisol through immersion (Sloman, 2010, Burton *et al.*, 2011) and injection (Best *et al.*, 2017). Many of these methods have serious limitations, making the observed results highly variable and context-specific. For example, exposure to acute stressors, even when repeated over a period of time, is unlikely to produce the same effects as chronic stress exposure. Cortisol implants are widely used as a proxy of chronic stress, but it is unclear how the release of hormones from these implants reflects biologically and ecologically relevant cortisol levels, in addition to the effects of the implantation process on the stress axis and offspring phenotype (Dufty *et al.*, 2002, Hoogenboom *et al.*, 2011, Crossin *et al.*, 2016). Lastly, exposure of ova to exogenous cortisol as a proxy of heightened maternal stress levels only provides information on the effect of cortisol, without consideration of the other factors that may link maternal stress and offspring phenotype, e.g. thyroid hormones (Ruuskanen and Hsu, 2018), androgens (Groothuis *et al.*, 2005) and mRNAs (Sopinka *et al.*, 2017).

In this study, I used three-spined sticklebacks in an attempt to shed some light on these uncertainties and disentangle the effect of relatively mild chronic maternal stress on offspring behaviour and stress physiology. I exposed adult females to an unpredictable chronic stress protocol, in which they experienced a range of environmental and husbandry stressors they could encounter in the wild and/or in captivity. I then subjected the offspring of these females to a number of behavioural assays and measured their hormonal response to an acute

stressor. The primary aim of this study was to determine whether maternal exposure to unpredictable and potentially stressful environmental conditions affected the behavioural phenotype of their offspring, with the secondary aim being to establish the link between stress hormone levels and behavioural measures in these offspring. In addition, I used a non-invasive method of water-borne cortisol quantification, which allowed me to make longitudinal measurements of glucocorticoid hormones in the same female throughout the breeding season. I was therefore able to investigate whether the potential behavioural and hormonal effects of chronic maternal stress differ between the offspring originating from successive clutches produced throughout a female's reproductive lifespan in a species where reproduction is mostly confined to a single breeding season.

Methods and materials

Study system

Fish used in this chapter were produced in the previous spring/summer through *in vitro* fertilisation of chronic stress-exposed (Experimental) and non-stress-exposed (Control) female three-spined sticklebacks. Maternal stress exposure and the procedure of fertilisation are described in detail in Chapter 2 and Chapter 3, respectively. Juvenile sticklebacks were kept in their family groups (reduced to 16 fry or less at day 60 post hatching, see Chapter 4) in clear 10 L plastic tanks (17x19x32 cm). Each tank was fitted with an air stone for aeration as well as a plastic plant and a piece of dark-coloured PVC pipe (cut in half) for shelter. Filtered and UV-sterilised water from a recirculating system (chilled to 12 °C) was continuously supplied to each tank. The fish were fed twice daily *ad libitum* with a commercial pellet (ZM Small Granular, ZM Systems). The tanks were siphoned on alternate days to remove excess food. Tanks containing the offspring of Experimental and Control females were randomly distributed throughout the aquarium room, controlling for artefacts due to spatial heterogeneity in the room.

Behavioural and physiological assays (see below) were restricted to the offspring of females that produced at least 2 clutches during the breeding season, to allow for a comparison between the clutches within each treatment and to account for potential confounding factors that resulted in any females being able to produce only one clutch during the breeding season. In addition, I used families with at least 3 surviving juveniles at the time of each assay to account for potential social aspects that could alter behaviour and stress physiology.

Behavioural tracking

To measure the effect of maternal stress exposure on various aspects of offspring behaviour, I performed behavioural observations of 215 individual fish from 73 families (35 Experimental and 38 Control families) using Lolitrack Quattro video tracking software (version 1.00, Loligo Systems) between 6th and 20th March 2018, on average 276 days post hatching (range: 243-306 days). Mean age of fish from the Experimental and Control group was 275 (range: 253-306) and 277 (range: 243-306) dph respectively. The setup for behavioural tracking consisted of four white opaque plastic arenas (24x17x10.5 cm), allowing tracking of up to four fish simultaneously (Figure 5.1a). Each arena was filled with 500

mL of aquarium water at 12 °C, so that the fish were fully submerged but unable to move up and down in the water column.

The setup was shielded with opaque black plastic on all four sides and illuminated from above by two 32cm LED lighting strips producing 50 lumens of luminous flux each. A web camera connected to a PC was fitted above the arena. Each set of behavioural observations consisted of the following trials, carried out for 10 minutes each in immediate succession:

1. Open field trial (OF) – fish were placed individually in an empty arena and their movement recorded
2. Novel object trial (NO) – an object with which the fish was not familiar (clear plastic ring approx. 3 cm in diameter) was lowered into the arena and the movement of the fish recorded
3. Sheltering trial (SH) – the novel object was removed from the arena, and a piece of PVC pipe with which the fish were previously familiar (since it came from their home tank) was placed in a corner of the arena. The area containing the shelter was masked from the view of the camera in the Lolitrack software, so that only movement of the fish outside of the shelter was recorded
4. Thigmotaxis trial (TH) – the shelter was removed, and an area in the centre of the arena (one body size from the perimeter of the arena on each side) was masked from the view of the camera in the Lolitrack software, so that only the movement of the fish around the perimeter was recorded

Figure 5.1b shows a schematic representation of the arena during each of the trials. The above set of observations was performed on three randomly selected fish per family, with the three fish measured on separate days. The minimum time period to complete the measurements for each family was 4 days and the maximum was 14 days. Following the trials, fish were placed in temporary tanks to avoid observing the same fish more than once. Water temperature was recorded after each set of trials (range recorded: 12.0-13.1 °C) and water replaced in the arenas.

Live tracking at a rate of one video frame per second was performed according to the software operation manual. All areas, with the exception of the arenas,

were masked from the view of the camera to avoid tracking other extraneous objects that had the same contrast as the fish. Contrast detection threshold was checked prior to each set of trials and adjusted manually where required. Live tracking during each trial yielded a data file containing x,y-coordinates (in pixels) of fish; unit calibration at the beginning of the experiment produced a spatial resolution of 0.086 mm per pixel. Custom R function (Cooper, unpublished) was used to convert these coordinates into the following information: distance travelled (in m) and the proportion of time spent moving in the OF trial, distance travelled (in m) and the proportion of time spent moving in the NO trial, proportion of time spent in the shelter in the SH trial and proportion of time spent at the perimeter of the arena in the TH trial.

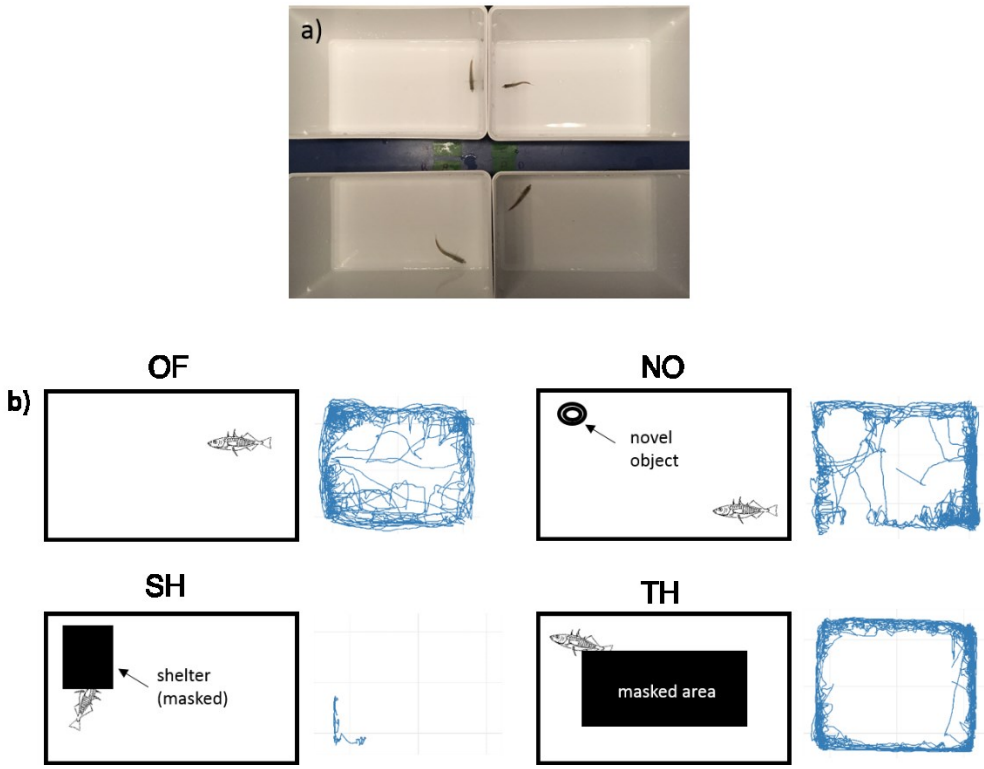

Figure 5.1 The arenas used for simultaneous tracking of the behaviour of four three-spined sticklebacks, as seen from above (a). Schematic representation of a single arena during the open field (OF), novel object (NO), sheltering (SH) and thigmotaxis (TH) trials, along with sample tracks produced in each of the trials (b). Masking in the SH and TH trials was performed in the video tracking software, so that when fish were in the masked area they were not tracked (but there was no effect of this masking on the behaviour of the fish).

Cortisol sampling and measurement

To test for differences in hormonal stress responses of the offspring of Experimental and Control females, between 9th and 12th April I exposed a total of 204 offspring to an acute stressor and collected water borne cortisol samples. The mean age of fish at the time of sampling was 305 days (range: 276-332 days); the mean age of fish from the Experimental and Control group was 308

(range: 284-326) and 304 (range: 276-330) days respectively. The non-invasive water sampling procedure was based on Sebire *et al*. (2007, 2009), with minor refinements resulting from pilot assays.

Each fish was exposed to air for 2 x 2 min, with a 2 min resting period in between the exposures. This has been established to induce acute stress response in zebrafish and sticklebacks (Ramsay *et al.*, 2009, Chapter 2 of this thesis). The fish were then placed individually in 600 mL borosilicate glass beakers filled with 100 mL of filtered and UV-sterilised water from the aquarium system for 90 minutes. The water samples were transferred to 50 mL polypropylene tubes and stored at -20 °C until further analysis. Between uses, the beakers were washed with distilled water and 100% methanol, and thoroughly dried.

Prior to cortisol extraction, the samples were defrosted and brought up to room temperature. Due to the project limitations, water samples from all fish from the same family were pooled and processed as one sample to obtain an average concentration per family. The samples were processed through individual solid-phase extraction cartridges (Sep-Pak C18 Plus Light, Waters), extracted with 100% methanol, diluted 1:3 in an assay buffer (supplied with the kit) and cortisol quantified using a colorimetric competitive ELISA assay (Enzo Life Sciences), with each sample being assayed in duplicate. This process of cortisol extraction and quantification is described in detail in Chapter 2. Samples with more than 19% CV between the duplicates (6 out of 73) were re-assayed; one of these samples had a CV of 34.4% after being re-assayed and was thus excluded from further analysis. Mean values of the intra- and inter-assay CV across three plates were 6.83% and 0.35% respectively. Mean extraction efficiency of a sample spiked with a known amount of cortisol (1.25 µL per mL) was 128%.

Data analysis

Pearson product-moment correlations were calculated between the four measurements obtained from the OF and NO trials (distance travelled and the proportion of time spent active in each trial), to determine whether these scores could be combined in a Principal Components Analysis (PCA). Table 5.1 shows the pairs of scores along with their correlation coefficients and associated p-values. SH and TH scores showed a non-normal distribution and were thus analysed separately.

Table 5.1 Pearson product-moment correlation coefficients between pairs of behavioural scores in the OF and NO trials: activity (proportion of time spent moving) in the open field (OF) trial, distance travelled in the OF trial, activity in the novel object (NO) trial and distance travelled in the NO trial.

Pair of scores	Corr. coeff	p-value
Activity OF – Distance OF	0.966	<0.001
Activity OF – Activity NO	0.732	<0.001
Activity OF – Distance NO	0.711	<0.001
Activity NO – Distance NO	0.968	<0.001
Activity NO – Distance OF	0.715	<0.001
Distance NO – Distance OF	0.739	<0.001

Since the four variables from the OF and NO trials were highly correlated (Table 5.1), they were summarised with PCA, after being z-score standardised using the "sapply" function in R Base Package (R Core R, 2017) to account for differences in standard deviation. The resulting single principal component (PC1) was used as an index of activity in further analyses. A linear mixed model (LMM) was used to analyse whether activity (PC1) differed between the offspring of Experimental (n=103 individuals) and Control (n=112) females, or between the offspring from the successive clutches produced during the breeding season (Clutch 1 n=84 individuals, Clutch 2 n=69, Clutch 3 n=62). The following fixed factors were included in the initial model: maternal treatment (categorical variable), clutch number (categorical) and size of the family group (covariate; group size ranged 4-16 individuals in the Experimental group and 3-15 individuals in the Control group), as well as the interaction maternal treatment *clutch number. In this and all subsequent models, Maternal ID was included as a random factor where multiple measurements (clutches) from the same female were used.

The TH score was arcsine-transformed and analysed separately using an LMM with the same fixed and random factors as in the activity model. Because the SH score distribution could not be normalised through transformation, I instead analysed the difference in the probability of sheltering depending on maternal treatment and clutch from which the fish originated. For the purpose of this analysis, the fish were divided into two groups: fish that used the shelter rather little during the trial (i.e. their sheltering proportion was between 0 and 0.49; termed 'non-sheltering' fish) and fish that sheltered extensively during the trial (sheltering proportion of 0.5-1.00; termed 'sheltering' fish). A generalized linear mixed model (GLMM) with binomial distribution was then used on this modified

dataset to predict the probability of a fish being classified as sheltering, with the same fixed and random factors as in the activity and TH model.

Similarity of the behavioural scores among related individuals, both within the clutches (i.e. for siblings) and amongst all of the offspring produced by each female regardless of the clutch (i.e. for individuals that were at least related as half-siblings, hereafter termed half-siblings), was analysed using "rptR" package in R using an approach that is usually used to calculate repeatabilities (Stoffel *et al.*, 2017). These scores were translated into an intra-family measure of variability of behaviour, where high similarity was associated with low variability between siblings or half-siblings, and *vice versa*.

A separate analysis was performed to determine whether offspring cortisol level following exposure to an acute stressor and their behaviour were correlated, and whether this correlation was dependent on maternal treatment and clutch number. As in the general analysis of behaviour, activity OF, distance OF, activity NO and distance NO scores were summarised with PCA whilst the probability of sheltering and the TH score were analysed separately. Due to the lack of data on offspring cortisol for several of the families, the sample size was smaller than in the original analysis: Experimental n=102 individuals from 34 families (Clutch 1 n=39 individuals, Clutch 2 n=33, Clutch 3 n=30), Control n=109 individuals from 37 families (Clutch 1 n=41 individuals, Clutch 2 n=36, Clutch 3 n=32). A LMM was used to analyse the activity (PC1), with mean cortisol (covariate; individual cortisol data were not available and therefore a mean value per family was used, which was the same for all three fish in the family) as a fixed factor and maternal ID as a random factor. Arcsine-transformed TH score was analysed using an LMM and the probability of sheltering was analysed using a GLMM with binomial distribution, with the same fixed and random factors as in the activity model.

To evaluate whether maternal baseline cortisol levels (see Chapter 2 for details) had an effect on offspring behaviour, regardless of maternal treatment and clutch number, I performed LMMs (for activity and arcsine-transformed TH score) and a GLMM with binomial distribution (for probability of sheltering). The factors included in the model were maternal baseline cortisol (covariate) and a random factor (maternal ID). Due to the limited data on maternal cortisol, the sample

size in these analyses was reduced compared to the previous analyses (n=155 individuals being the offspring of 20 females).

The MyAssays online data analysis tool (MyAssays Ltd.) was used to calculate offspring peak cortisol concentrations from optical densities, using the Four Parameter Logistic Curve as per the kit manufacturer's instructions. A linear model (LM) was used to analyse the difference in mean peak cortisol levels between the families produced by Experimental (n=34) and Control (n=37) females and between the offspring from the successive clutches produced during the breeding season (Clutch 1 n=27, Clutch 2 n=23, Clutch 3 n=21). The following fixed factors were included in the initial model: maternal treatment (categorical variable), clutch number (categorical) and size of the family group (covariate), as well as the interaction term maternal treatment *clutch number. Initially, Maternal ID was included as a random factor. However, the variance of the random effect was estimated as 0 (resulting in a singular model fit) and it was thus dropped without affecting model estimates.

LMs were used to analyse the relationship between offspring acute cortisol and maternal post-experimental baseline cortisol, and between offspring acute cortisol and maternal acute cortisol, regardless of maternal treatment and clutch number. Both maternal and offspring cortisol were log-transformed to achieve normality of the residuals, and log-transformed maternal cortisol was used as a fixed factor in the models. As in the model above, the random factor (Maternal ID) was dropped as it led to model singularity.

All statistical analyses were performed in R statistical software (version 3.4.3, R Development Core Team), with LMMs fitted using the "lme4" package in R (Bates *et al.*, 2015). The degrees of freedom were estimated by Satterthwaite approximation (for normally distributed data) and Laplace approximation (for binomial data). P values were obtained using the "lmerTest" package, with an alpha level of 0.05 (Kuznetsova *et al.*, 2017). For all models, non-significant terms were removed by backwards selection.

Results

Behavioural scores

The first principal component (PC1) extracted from the PCA of activity OF, distance OF, activity NO and distance NO scores, had an Eigenvalue of 1.85 (and thus satisfied the Kaiser-Guttmann criterion of Eigenvalue > 1) and explained 85% of the total variance in the data (loadings of the four individual variables: activity OF = -0.499, distance OF = -0.501, activity NO = -0.500, distance NO = -0.500). Higher PC1 scores thus indicate higher overall activity levels, with fish spending more time in motion and covering a greater total distance during the trial. Bartlett's test of sphericity ($p<0.001$) and the Kaiser-Meyer-Olkin (KMO) test of sampling adequacy (0.52) indicated that a minimum standard was met to proceed with the results of the PCA. However, the results should be treated with caution since KMO results between 0.5 and 0.6 are considered poor but acceptable (Kaiser and Rice, 1974). There was no significant difference in activity levels and thigmotaxis between offspring of Experimental and Control females, nor between offspring from the successive clutches produced over a breeding season; the interactions between treatment and clutch number were also non-significant, as were the size of the family group (Table 5.2). Activity score (PC1) was similar for both siblings and half-siblings in the Control but not in the Experimental group (Table 5.3, Figure 5.2). Thigmotaxis showed very low repeatability in both treatment groups.

The probability of a fish being classified as sheltering was independent of maternal treatment and the size of the family group. There was a tendency for the juveniles hatched later in the breeding season (Clutch 3) to have a higher probability of being classified as sheltering, but this was only marginally significant (Figure 5.3, Table 5.2). Sheltering behaviour was found to be repeatable in the Control but not in the Experimental group (Table 5.3).

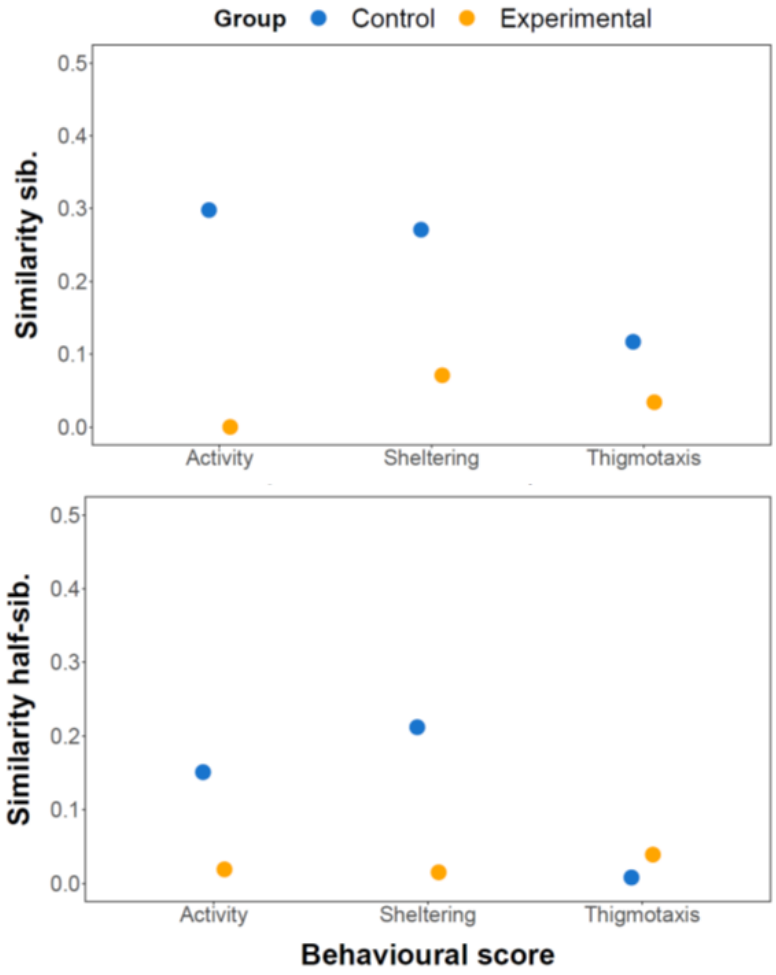

Figure 5.2 Similarity coefficient of the behavioural scores (activity (PC1), probability of sheltering and thigmotaxis) in juveniles produced by three-spined stickleback females from Control (n=112; blue) and Experimental (n=103; orange) treatment groups. The top panel represents similarity in behaviour amongst full siblings within a clutch; the bottom panel represents the similarity amongst all of the offspring produced by each female (half-siblings). The activity level (PC1) was obtained from a PCA of the four following scores: distance travelled and the proportion of time spent active in both the open field and novel object trials.

The PCA on the reduced dataset, used to analyse the relationship between offspring behaviour and their peak cortisol level, yielded a PC1 (activity score) with an Eigenvalue of 1.84, which explained 85% of the total variance. Factor loadings, as well as the Bartlett's and KMO test results were the same as in the previous PCA. Activity (PC1; $t_{1,\ 187.54}$=-1.201, p=0.231), probability of sheltering (z=-0.334, p=0.739) and TH score ($t_{1,\ 167.23}$=-0.857, p=0.393) were independent of the peak cortisol level of the offspring. In addition, maternal baseline cortisol did not predict offspring activity (PC1; $t_{1,\ 13.22}$=1.426, p=0.177), probability of sheltering (z=0.050, p=0.960) or TH score ($t_{1,\ 16.66}$=-0.331, p=0.745).

Table 5.2 Summary of Linear Mixed Models (LMM) used to analyse the differences in activity, sheltering probability and thigmotaxis (TH; arcsine-transformed) in juveniles from three successive clutches produced by Control and Experimental female three-spined sticklebacks during the breeding season. Values give estimates ± s.e. The reference maternal treatment in all models was the Control group, and the reference clutch was the first clutch in the season. Maternal ID was included in the models as a random factor. Control group n=112 (Clutch 1 n=44, Clutch 2 n=36, Clutch 3 n=32) and Experimental group n=103 (Clutch 1 n=40, Clutch 2=33, Clutch 3=30). Maternal treatment, Clutch number and all significant terms (indicated with p-values in underlined and bold font) were retained in the final models.

Model terms	Effect on activity (PC1)	Effect on shelt. prob.	Effect on TH score
Maternal treatment	-0.326 ± 0.309, $t_{1,21.60}$=-1.055, p=0.303	-0.082 ± 0.405, z=-0.203, p=0.839	-0.010 ± 0.023, $t_{1,27.95}$=-0.453, p=0.654
Clutch 2	0.285 ± 0.293, $t_{2,195.89}$=0.975, p=0.331	0.232 ± 0.351, z=0.660, p=0.509	-0.034 ± 0.025, $t_{2,201.93}$=-1.362, p=0.175
Clutch 3	-0.086 ± 0.303, $t_{2,199.02}$=-0.284, p=0.776	0.734 ± 0.374, z=1.961, **p=0.050**	-0.005 ± 0.026, $t_{2,204.61}$=-0.199, p=0.842
Mat. treatment *Clutch 2	-0.558 ± 0.590, $t_{2,193.92}$=-0.946, p=0.345	0.205 ± 0.701, z=0.292, p=0.770	0.047 ± 0.055, $t_{2,199.50}$=0.932, p=0.352
Mat. treatment *Clutch 3	0.011 ± 0.610, $t_{2,195.75}$=0.018, p=0.986	0.368 ± 0.745, z=0.494, p=0.621	0.047 ± 0.052, $t_{2,201.30}$=0.857, p=0.392
Family group size	0.014 ± 0.038, $t_{1,188.35}$=0.364, p=0.716	0.076 ± 0.047, z=1.595, p=0.111	-0.000 ± 0.003, $t_{1,175.50}$=-0.262, p=0.794

Table 5.3 Similarity coefficient of behavioural scores within the clutches (siblings, S) and across all of the offspring (half-siblings, HS) produced by each Experimental (n=14) and Control (n=16) female three-spined stickleback. Numbers in brackets denote standard error of the mean. Significant similarity is indicated with p-values in underlined and bold font. Sample sizes: Experimental n=103 individuals from 35 clutches, Control n=112 individuals from 38 clutches.

	Activity (PC1)		Sheltering prob.		Thigmotaxis	
	S	HS	S	HS	S	HS
Overall	0.131 (0.071), p=0.050	0.086 (0.053), p=0.051	0.201 (0.076), **p=0.004**	0.114 (0.059), **p=0.005**	0.065 (0.063), p=0.196	0.023 (0.033), p=0.316
Experimental	0.000 (0.065), p=0.500	0.019 (0.045), p=0.448	0.071 (0.079), p=0.221	0.015 (0.040), p=0.383	0.034 (0.073), p=0.405	0.039 (0.056), p=0.293
Control	0.298 (0.107), **p=0.003**	0.151 (0.086), **p=0.027**	0.271 (0.131), **p=0.009**	0.212 (0.113), **p=0.002**	0.117 (0.094), p=0.142	0.008 (0.043), p=0.500

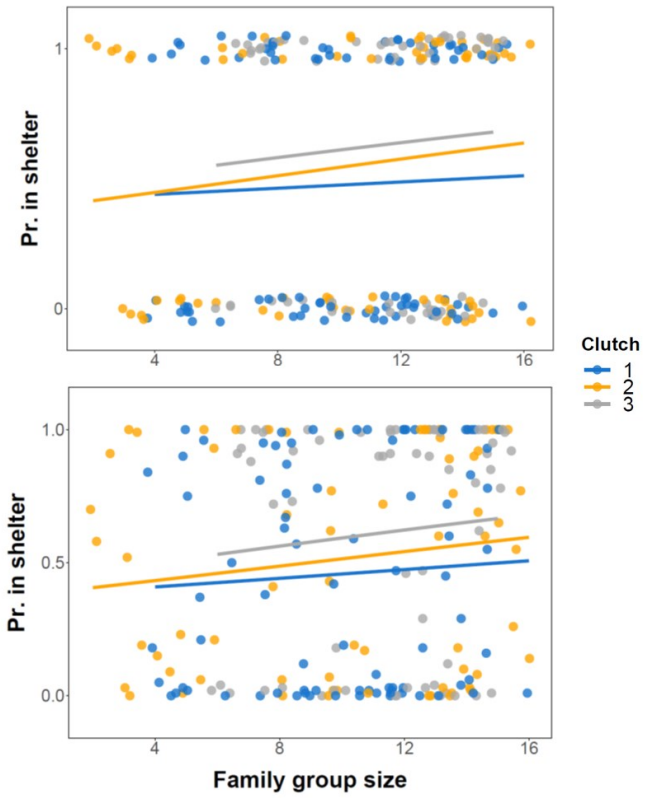

Figure 5.3 The relationship between the clutch from which the fish originated (Clutch 1 n=84, blue; Clutch 2 n=69, orange; Clutch 3 n=62, grey), the size of the family group and the sheltering score. The top panel shows the probability of a fish being classified as sheltering, with the probability being assigned 0 if the proportion of time sheltering during the trial was between 0 and 0.49 and the probability being assigned 1 if the proportion of time was between 0.5 and 1. The points have been offset for clarity of the presentation. The bottom panel shows the original data from which these probabilities were derived.

Cortisol response to an acute stressor

Offspring of the females subjected to a period of chronic stress did not directly show an altered hormonal response following the exposure to an acute stressor (Table 5.4). Overall, juveniles from clutches produced later in the breeding season (Clutch 3) had higher peak cortisol levels. There was also an indication that maternal stress treatment may have altered inter-clutch differences in peak cortisol level, with juveniles from Clutch 3 produced by stress-exposed mothers tending to release less cortisol in response to an acute stressor (Figure 5.4, Table 5.4). The size of the family group did not affect peak cortisol levels. In addition, neither maternal baseline cortisol level ($t_{1, 48}$=1.175, p=0.246), nor the peak cortisol produced by mothers in response to an acute stressor exposure ($t_{1, 48}$=-0.351, p=0.727) predicted the hormonal response of the offspring to an acute stressor.

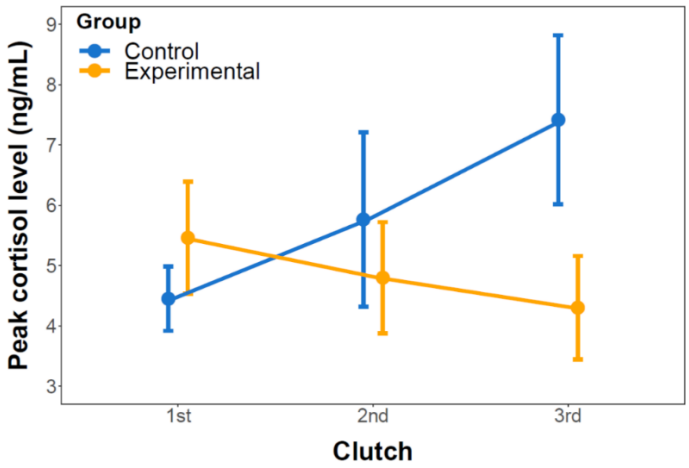

Figure 5.4 Differences in mean peak cortisol levels following the exposure to an acute stressor (in ng/mL) in juveniles from three successive clutches produced across the breeding season by three-spined stickleback females from Control (n=37; Clutch 1 n=14, Clutch 2 n=12, Clutch 3 n=11; blue) and Experimental (n=34; Clutch 1 n=13, Clutch 2=11, Clutch 3=10; orange) treatment groups. The error bars represent Standard Error of the Mean (s.e).

Table 5.4 Summary of the Linear Model (LM) used to analyse the variation in peak cortisol level (in ng/mL) in juveniles from three successive clutches produced by Control and Experimental female three-spined sticklebacks during the breeding season. Values give estimates ± s.e. The reference maternal treatment was the Control group, and the reference clutch was the first clutch in the season. Control group n=37 (Clutch 1 n=14, Clutch 2 n=12, Clutch 3 n=11) and Experimental group n=34 (Clutch 1 n=13, Clutch 2=11, Clutch 3=10). Maternal treatment, Clutch and all significant terms (indicated with p-values in underlined and bold font) were retained in the final model.

Model terms	**Effect on offspring peak cortisol**
Maternal treatment	1.010 ± 1.384, $t_{1,70}$=0.730, p=0.468
Clutch 2	1.314, ± 1.413, $t_{2,70}$=0.930, p=0.356
Clutch 3	2.967 ± 1.447, $t_{2,70}$=2.050, **p=0.044**
Mat. treatment *Clutch 2	-1.976 ± 2.040, $t_{2,70}$=-0.968, p=0.336
Mat. treatment *Clutch 3	-4.126 ± 0.008, $t_{2,70}$=2.092, p=0.053
Family group size	-0.132 ± 0.125, $t_{1,70}$=-1.058, p=0.294

Discussion

Behavioural phenotype

The results of this study provide no direct and straightforward evidence of maternal adjustments to offspring behavioural phenotype following chronic stress exposure, with none of the three behavioural scores (activity, probability of sheltering and thigmotaxis) showing any significant relationship with maternal treatment if offspring behavioural phenotype is treated simply as a consequence of maternal stress. These results are at odds with the current evidence from a range of species and taxa, where maternal stress effects (or pre-natal exposure to exogenous GCs as a proxy of maternal stress) tend to be apparent in terms of offspring behaviour, although the direction of these effects may vary between contexts.

For example, exposure of mothers to predators or predator cues has been shown to result in increased activity and boldness of their offspring (Archard *et al.*, 2012, Bestion *et al.*, 2014), as did the exposure of ova to exogenous GCs (Colson *et al.*, 2015, Sopinka *et al.*, 2015a, Best *et al.*, 2017). Conversely, maternal cortisol treatment has been reported to result in reduced offspring activity in general (Redfern *et al.*, 2017) and under various scenarios, such as confinement (Eriksen *et al.*, 2011) and encounter with a novel environment (Espmark *et al.*, 2008). Exposure of mothers to physical stressors has also been shown to affect offspring activity, either positively (O'Brien *et al.*, 2017) or negatively (Colson *et al.*, 2019), with the nature and duration of stressors likely to have an impact on the direction of this effect.

Other aspects of offspring behaviour may also be affected by maternal stress. The propensity to remain close to the walls whilst in an open space (thigmotaxis) is used as an indicator of anxiety levels (Simon *et al.*, 1994), and it tends to be negatively correlated with maternal stress/exogenous GC exposure (Baiamonte *et al.*, 2016, Best *et al.*, 2017, Redfern *et al.*, 2017, but see Kapoor and Matthews, 2005, who provide an evidence of increased offspring anxiety in response to maternal stress). Sheltering behaviour, associated with the avoidance of predators and unfavourable environmental conditions, as well as energy conservation (Kerry and Bellwood, 2017), often increases in organisms exposed to pre-natal stress (Uller and Olsson, 2006, Ensminger *et al.*, 2018).

A possible explanation of the lack of chronic maternal stress effect observed in the present study may be related to the mechanism through which mothers adjust offspring behaviour in response to stress. Here, I found evidence of behavioural but not hormonal responses of females to chronic stress (Chapter 2), and no evidence of stress-exposed females depositing more cortisol in their eggs than controls (Chapter 3). As indicated by some of the above examples, elevated stress hormone levels may drive the change in offspring behavioural phenotype. Therefore, if the offspring sticklebacks did not experience elevated cortisol levels *in ovo*, their behavioural phenotype may be independent of maternal stress. The validity of this explanation is further supported by the observed lack of correlation between offspring behavioural scores and maternal baseline cortisol level, regardless of whether the mothers were exposed to a period of chronic stress or not.

In addition, there is some recent evidence that in certain situations the behaviour of the offspring may be buffered from the effects of maternal stress, regardless of what method is used to induce these effects (Cortez Ghio *et al.*, 2016). The adjustment of offspring phenotype may also depend on the nature of the environment experienced by mothers. In a stressful but relatively stable environment, e.g. where high predation levels are present, it may be beneficial for mothers to alter the behavioural response of their offspring to predators. However, in an unpredictable environment such as was produced by the stress protocol used in this study, the cost of adjusting the behavioural phenotype may outweigh the benefits, for example if the alteration of behaviour comes at a cost to growth or survival (Groothuis *et al.*, 2005, Sheriff and Love, 2013b, Sheriff *et al.*, 2018).

Even though the offspring behavioural phenotype did not seem to differ directly between the offspring of Experimental and Control females, exposure of mothers to a period of chronic stress increased the variability of behavioural traits (with the exception of thigmotaxis) in their offspring, an effect that was consistent both within and between the breeding attempts (clutches) of the same female. Whilst repeatability of behaviour within an individual is well documented, including in the context of maternal effects (Bell *et al.*, 2009, Reddon, 2012), rather few studies have quantified the similarity of behavioural phenotypes across individuals of the same family, particularly in response to maternal stress. However, my results are congruent with studies on zebra finches (Careau *et al.*,

2014) and convict cichlids (Oldham *et al.*, 2019), which also show increased intra-family variation in behaviour in response to environmental perturbation. It remains unclear whether the reduced similarity in offspring behaviour within families is an example of an adaptive maternal effect, but it may be an attempt to increase offspring fitness in unpredictable environment by producing individuals differing in behavioural phenotype, and thus increasing the chance that some of these individuals will be able to better cope with the prevailing conditions.

The behaviour of offspring in the present study was largely independent of the clutch from which they originated, with no difference between early- and late-produced clutches. The exception was sheltering behaviour, with late-produced offspring showing a small but statistically significant increase in the probability of using a shelter. It is, however, not possible to say whether the observed effect resulted from adaptive maternal adjustment of offspring sheltering behaviour, e.g. to increase the survival probability of the offspring produced late in the breeding season. There is also a lack of comparable studies looking at inter-clutch differences in fish behaviour; thus, it requires further experimental investigation to determine whether the observed difference in sheltering behaviour among clutches is a robust and ecologically relevant effect.

Despite the available evidence of a group-size effect on animal behaviour (Schleuter *et al.*, 2007, McClure *et al.*, 2009, Middlemiss *et al.*, 2018), in this study there was no relationship between family group size and any of the three measured aspects of behaviour. The possible explanations for this result include the relatively small difference between the biggest (16 individuals) and the smallest (3 individuals) group size, as well as an *ad libitum* feeding protocol and relatively large size of the tanks, both of which had the potential to reduce competition and density effects on stickleback behaviour (Ward *et al.*, 2006, Brockmark *et al.*, 2010, Johnsson and Naslund, 2018).

In addition to being influenced by non-genetic maternal effects, behavioural traits tend to be moderately to highly heritable (Ariyomo *et al.*, 2013, Ferrari *et al.*, 2016, Edwards *et al.*, 2017, see also the metaanalysis by Stirling *et al.*, 2002) and thus it would be expected that offspring behaviour reflects that of their mother. To be able to reliably compare maternal and offspring behaviours, these should be measured on an individual scale and in the same context (Koski,

2014, Roche *et al.*, 2016). Due to the technical and procedural limitations of my study, it was impossible to measure the same behavioural traits in mothers and offspring, and the behaviour was scored at a treatment (mothers) and family (offspring) rather than an individual level. However, the similarity of behavioural scores amongst half-siblings (albeit most prominent in the offspring of non-stressed exposed females) suggests that the observed behavioural phenotypes are likely to be a product of maternal or genetic effects rather than being generated at random. Nonetheless, the extent to which offspring behaviour reflects maternal behaviour, and how this is modified by maternal chronic stress exposure, should be addressed in future studies using methods that make the results comparable and interpretable.

Hormonal stress response

The hormonal response to an acute stressor of juvenile sticklebacks produced by stress-exposed mothers did not differ from that of controls. The results of this study are therefore incongruent with the evidence from fish and other taxa, where maternal stress exposure had an effect on offspring stress physiology. For example, maternal stress can result in increased (Bian *et al.*, 2015) or reduced (Tilgar *et al.*, 2016, Kleist *et al.*, 2018) baseline cortisol levels of the offspring, depending on the context and the nature of the stressor. Furthermore, pre-natal stress exposure can have an effect on the development and reactivity of the stress axis, with offspring hormonal response to stressors having a tendency to be attenuated as a result of maternal stress (Baiamonte *et al.*, 2016, Jeffrey and Gilmour, 2016, Nesan and Vijayan, 2016, Redfern *et al.*, 2017, Sopinka *et al.*, 2017). Even when the magnitude of the stress response is unaffected, the time course of stress hormone release may be altered (Mommer and Bell, 2013, Tilgar *et al.*, 2016); thus in future studies it would be beneficial to measure offspring hormonal responses at different time points after the acute stress exposure. It is unclear whether the lack of correlation between maternal stress exposure and offspring stress physiology observed in this study reflects an adaptive maternal strategy or arises because relatively mild chronic maternal stress has no effect on the development of the stress axis of the offspring. The former would explain my results if anticipated unpredictable offspring environment favoured greater flexibility of physiological stress responses.

Despite showing no direct effect of maternal stress treatment, the results of this study indicate that mothers experiencing chronic stress throughout the breeding season (which is equivalent to throughout their reproductive lifespan, as in this annual stickleback population; (Lee *et al.*, 2012)) may differentially adjust the physiological responses to stress of the offspring from different clutches. Whilst overall the offspring from clutches produced late in the breeding season showed significantly higher peak cortisol levels after an acute stress exposure, this seems to be driven mainly by the offspring of the Control females. When the interaction between maternal treatment and clutch number is taken into account, there is suggestive evidence that chronic maternal stress drives down the peak cortisol values in late-produced clutches. Since GCs tend to have a pleiotropic effect on various aspects of animal physiology (McGlothlin and Ketterson, 2008, Hau and Goymann, 2015), it would be reasonable to expect that females anticipating stressful future conditions modify the reactivity of their offspring to stressful stimuli if high reactivity is negatively correlated with other aspects of the phenotype, such as growth rate (Marshall and Uller, 2007). This would be particularly important in the late-produced offspring, which have a limited time to reach a size that maximises their overwinter survival potential, particularly if the environment is stressful and unpredictable.

Contrary to the expectations stemming from published studies on the relationship between the size of the social group, GC levels (Michelena *et al.*, 2012, Robertson *et al.*, 2017) and hormonal response to stressful stimuli (Mommer and Bell, 2013, Emmerson and Spencer, 2018), in this study the size of the family group had no effect on peak cortisol levels following acute stress exposure. As with the behavioural scores, this may result from the lack of sibling competition and density effects (Bolasina *et al.*, 2006, Rensel *et al.*, 2011) due to the nature of the housing and feeding protocol. In addition, factors such as the size of group members (Ligocki *et al.*, 2019) and a social hierarchy within the group (Gilmour *et al.*, 2005) may play an important role in determining the magnitude of group size effect on hormonal stress response, and these should be taken into account in the future studies.

As with behavioural phenotypes, hormone levels show a moderate to high degree of heritability (Okuliarova *et al.*, 2011, Jenkins *et al.*, 2014); this is also true for the GC response to stressors (Fevolden *et al.*, 1999, Volckaert *et al.*, 2012, Vandeputte *et al.*, 2016, Bairos-Novak *et al.*, 2018). Therefore, it would be

reasonable to expect that the peak cortisol response of the offspring would reflect that of their mother, regardless of maternal treatment. It is unclear why this was not the case in the present study, where the peak cortisol level of the offspring was independent of maternal cortisol level, both baseline and after an acute stress exposure. It is possible that pooling of the offspring cortisol samples led to the loss of valuable information, for example if the hormonal response was highly dependent on size or sex of the individuals; it would therefore be recommended to investigate the relationship between maternal and offspring cortisol in terms of individual offspring rather than a mean family value.

In the present study I did not observe any relationships between the peak cortisol in offspring and their behavioural phenotype, measured as activity, anxiety levels (the latter expressed as thigmotaxis) or sheltering behaviour, regardless of maternal treatment. Hormones, including GCs, are responsible for coordinating various aspects of an animal phenotype, including behavioural phenotype (McGlothlin and Ketterson, 2008, Hau and Goymann, 2015, Garland *et al.*, 2016). It has been demonstrated that GC levels are correlated with such aspects of animal behaviour as overall activity (Raoult *et al.*, 2017, Dender *et al.*, 2018), exploratory behaviour (Lawrence *et al.*, 2018a, Moyers *et al.*, 2018) and risk aversion (Baugh *et al.*, 2017). However, the hormonal response to an acute stressor is dependent on factors such as the nature of the stressor, reactivity of the stress axis, social environment, etc., and therefore baseline rather than the peak cortisol level may be more likely as a predictor of behaviour. Since I measured only the peak cortisol level in the offspring, it is difficult to draw conclusions about the lack of correlation between cortisol and behavioural scores in sticklebacks, and this question should be addressed in future studies.

To conclude, the analysis of the effect of chronic maternal stress on offspring behavioural phenotype and physiological response to an acute stressor showed that juvenile sticklebacks mothered by stress-exposed females did not differ in their behavioural scores, nor in their peak cortisol levels, from the offspring of the control females. However, I found an effect of maternal stress exposure on among-individual differences in behaviour, both within and between clutches, with chronic maternal stress reducing the similarity of behaviour between siblings and half-siblings. This finding provides the ground for further studies on maternal stress and the similarity of offspring behavioural traits between related

individuals, which are currently scarce. Both behavioural scores and the hormonal stress response were independent of the size of the family group, and no relationship was detected between maternal baseline cortisol and offspring behaviour. Moreover, cortisol level in the offspring did not reflect that of the mother and no link was observed between peak cortisol level and behavioural phenotype. In this study, I also attempted to bridge the existing knowledge gap by investigating how chronic maternal stress affects offspring from different reproductive attempts of a female. With the exception of the probability of sheltering, which showed a slight increase in late-produced clutches, I found no inter-clutch differences in behaviour, either in interaction with maternal stress treatment or independently. I did however find evidence that juvenile sticklebacks from successive clutches produced over the breeding season (which is equivalent to a female's reproductive lifespan) differed in their physiological response to an acute stressor, an effect that may potentially be modified by maternal exposure to chronic stress. Whilst the observed effect was relatively weak, it provides a basis for further studies of inter-clutch differences in stress physiology of iteroparous fish.

Chapter 6: The effects of chronic stress on offspring reproductive strategies and grandoffspring pre-natal development

Abstract

Stressful conditions experienced by individuals not only have the potential to shape their own phenotype, but can also have implications for the phenotype of their offspring. For example, if exposure to stressors affects female reproductive strategy by inducing mothers to invest more resources in their developing offspring, these offspring may themselves have an altered reproductive strategy. Therefore the effects of environmental stress experienced by a single generation may persist into future generations. However, little is known about transgenerational effects of chronic and relatively mild stress experienced by females (as opposed to acute maternal stress or exposure of developing offspring to exogenous GCs), particularly in vertebrate models.

In this study, I used three-spined sticklebacks to examine whether the effects of exposure to chronically stressful and unpredictable environmental conditions persist or manifest themselves further down the maternal line. I exposed F0 females to a period of chronic stress throughout the breeding season and reared their offspring from three successive clutches. I then examined the reproductive strategy of non-stressed females from the F1 generation and the pre-natal developmental trajectories of the F2 generation.

The daughters of stress-exposed F0 females produced smaller clutches consisting of heavier eggs, but this was only apparent in females of relatively high body mass. I also observed an egg mass-dependent increase in egg density in these females. In addition, there was an interaction between F0 stress treatment and the order of F0 spawning, with late-spawned daughters of stress-exposed females producing heavier eggs and offspring that were larger at hatching. Whilst the observed relationships are complex and context-dependent, they suggest that females faced with stressful environmental conditions may pre-program their daughters to invest more in reproduction. This intergenerational effect is possibly mediated by increased reproductive investment of F0 females, particularly towards the end of their reproductive lifespan.

Introduction

Non-genetic maternal influences have long been considered a major force shaping animal phenotypes. Factors such as maternal age, size, nutritional and social status, and the choice of nest site can have a strong influence on offspring physiology, morphology, behaviour and life-history (Mousseau and Fox, 1998, Wolf and Wade, 2009), either as a result of adaptive maternal programming of offspring phenotype or as a side effect of the maternal phenotype, maternal environment or the combination of both (Heath and Blouw, 1998, Sheriff and Love, 2013a, Sheriff and Love, 2013b). The adaptive potential of maternal effects can be highly dependent on the matching of the maternal-offspring environment, particularly where females adjust the phenotype of their offspring to provide them with a fitness advantage in the anticipated future environment (Marshall and Uller, 2007, Hoyle and Ezard, 2012). For example, females living in unpredictable or stressful environments may attempt to produce offspring with phenotypic characteristics allowing them to better cope with anticipated adverse environmental conditions (Sheriff and Love, 2013b, Sheriff *et al.*, 2018, but see Uller *et al.*, 2013).

There are many mechanisms through which non-genetic maternal effects, including the effects of maternal stress, can affect the offspring phenotype, from hormones (Groothuis *et al.*, 2005, Dantzer *et al.*, 2013, Ruuskanen and Hsu, 2018), nutrients (Griffith and Buchanan, 2010) and maternally-derived mRNAs (Adrian-Kalchhauser *et al.*, 2018, Colson *et al.*, 2019) to epigenetic modifications involving differential patterns of DNA methylation (Cameron *et al.*, 2008, Guerrero-Bosagna *et al.*, 2018). The latter may be of particular importance in the study of inter- and transgenerational change due to the high heritability of epigenetic modifications (Trerotola *et al.*, 2015). As a result, the phenotype and/or environment experienced by a mother have the potential to affect not only her offspring but also later generations. Indeed, transgenerational change (i.e. that affecting at least the F2 generation) due to stressful environment experienced by females encompasses more than just maternal effects (Burton and Metcalfe, 2014), and can have serious implications for the dynamics of populations and for their evolutionary potential (Benton *et al.*, 2005, Love *et al.*, 2013, Bian *et al.*, 2015, Sheriff *et al.*, 2017).

A situation in which there can be far-ranging inter- and transgenerational effects arises when a female's state affects her reproductive decisions or investment. This can shape the life history and reproductive strategy of her offspring (F1 generation), for example in terms of their size and sexual traits (Murphy *et al.*, 2014, Wilson *et al.*, 2019, Warburton *et al.*, 2019), reproductive function (Connor *et al.*, 2012), fecundity (Gorman and Nager, 2004) and reproductive investment (Sorci and Clobert, 1995, Guibert *et al.*, 2013, Langen *et al.*, 2019). These in turn can influence the development, survival and reproductive success of her grandoffspring (the F2 generation). Thus the effects of environmental factors (e.g. stress) acting upon the F0 generation can be carried over to the subsequent generations, even when these generations are not directly affected by the initial conditions.

Much of the current evidence of such effects comes from studies on invertebrates. This may be largely due to their short generation time, which allows for tracking reproductive strategies (and their fitness consequences) of many generations in a relatively short timespan. Some examples amongst invertebrate taxa of changes to life history and reproduction due to grandmaternal effects (e.g. following exposure of the F0 generation to variable diet quality or temperature) include reduced fecundity (Goos *et al.*, 2019), reduced developmental rate (Magiafoglou and Hoffmann, 2003), increased egg mortality (Jann and Ward, 1999) and reduced hatch rate (Kyneb and Toft, 2006). In addition, transgenerational environmental effects on invertebrate reproduction have been shown to be dependent on non-environmental factors, such as maternal age (Hercus and Hoffmann, 2000, Goos *et al.*, 2019), and to be compensated for if the maternal generation does not experience the same environmental conditions as the grandmaternal generation (Mikulski and Pijanowska, 2017, Crocker and Hunter, 2018). There is also increasing evidence from vertebrates that environmental factors acting upon parents may project into subsequent generations through the alteration of the life history and reproductive output of their offspring. This seems to be a general process spanning all vertebrate taxa and described in various contexts in mammals (Connor *et al.*, 2012, Bian *et al.*, 2015, Warburton *et al.*, 2019), birds (Gorman and Nager, 2004, Naguib *et al.*, 2006, Langen *et al.*, 2019), reptiles (Sorci and Clobert, 1995) and fish (Murphy *et al.*, 2014, Shama and Wegner, 2014, Le Roy *et al.*, 2017).

However, the adaptive potential of transgenerational effects spanning multiple generations remains unclear, mainly due to their high context-dependence. For example, unfavourable environmental conditions experienced by females are often associated with reduced reproductive success of their daughters, but there are instances in which this effect is positive and improves the breeding success of the offspring and quality of the grandoffspring (Goetz *et al.*, 2008, Ancona and Drummond, 2013), as well as situations where no effect is observed on offspring reproduction (Rutkowska *et al.*, 2007, Fraz *et al.*, 2019). Moreover, the strength and direction of transgenerational effects may be highly dependent on the stability of the environment, i.e. whether the environment experienced by daughters matches that of the mothers, in which case the effect may be cumulative (Shama and Wegner, 2014, Le Roy *et al.*, 2017, Warburton *et al.*, 2019).

Furthermore, there are currently limitations as to the environmental factors that have been used to study grandmaternal effects: whilst the effects of temperature, diet quality and social environment are widely represented in the literature, relatively little is known about the transgenerational effects of stress in general and chronic unpredictable stress in particular. However, these can be of paramount importance for the dynamics and persistence of populations in disturbed or unpredictable environments, even if the stressful conditions are only experienced by specific cohorts within a population. It is also unclear how stressful environmental conditions experienced by F0 females interact with the timing and order of their reproductive attempts in shaping of the F1 female reproductive strategy and pre-natal development of the F2 generation.

In this study, I explored these questions by exposing female three-spined sticklebacks to unpredictable chronic stress across the entire breeding season, during which they produced up to three clutches. The resulting offspring were reared in a non-stressful environment and allowed to breed in the following year. I was therefore able to examine whether chronically stressful conditions experienced by females from the F0 generation affected the reproductive strategy of their daughters (F1 generation) and the developmental trajectories of their grandoffspring (F2 generation). The design of this study, encompassing multiple reproductive attempts of an F0 female within a single breeding season, also allowed me to address the interaction between unpredictability of the environment and clutch order in shaping the F1 reproductive phenotype.

Methods and materials

Experimental setup and breeding

To analyse whether chronic stress experienced by female three-spined sticklebacks from the F0 generation affected the reproductive strategy of their daughters (F1 generation) and pre-natal developmental trajectories of their grandoffspring (F2 generation), I used as F1 fish the fry originating from the 2017 breeding season (described in Chapter 3). These fish were produced in the first, second or third reproductive attempt of females that either were subjected to an unpredictable chronic stress protocol (UCSP) throughout the breeding season (Experimental group) or had experienced standard husbandry procedures only (Control group; Figure 6.1). Only the F1 families with at least 3 fish that survived to sexual maturity were included in this experiment. This resulted in the following sample sizes: 13 F1 full-sib families for the Experimental group came from an F0 female's first clutch, 12 from the second clutch and 10 from the third clutch (from 14 F0 UCSP females); the corresponding sample sizes for the first, second and third clutch in Control group families were 15, 12 and 11 (from 16 F0 Control females). The details of the maternal stress treatment and breeding protocol are provided in Chapter 2.

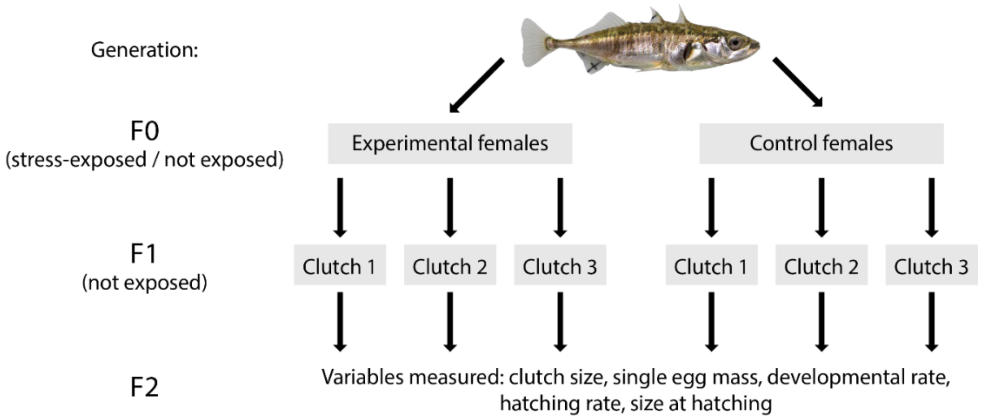

Figure 6.1 Schematic representation of the experimental setup. F0 females from the Experimental group were exposed to a period of unpredictable chronic stress protocol (UCSP), whilst F0 fish from the Control group and those from the F1 generation experienced only standard husbandry practices. Only the first clutch produced by the F1 females originating from each of the F0 females' breeding attempts (Clutch 1, Clutch 2 and Clutch 3) is used for the analysis of traits in the F2 generation.

The F1 fish were kept in their original hatching tanks (10 L plastic tanks, 17x19x32 cm) in family groups of up to 15 individuals. Each tank was fitted with an air stone for aeration and a plastic plant for sheltering, and constantly supplied with filtered, de-chlorinated and UV-sterilised water. Throughout the 2017-18 winter the tanks were kept at 12 °C and a photoperiod of 10L : 14D,

created by artificial lights. From 1st March 2018, the water temperature was gradually increased until it reached approximately 15 °C and the photoperiod was gradually changed to 14L : 10D. From five weeks post hatching, the fish were fed once daily *ad libitum* with commercial granular food (ZM Small Granular, ZM Systems); from 1st April 2018 they also received live *Artemia* nauplii once daily as a supplementary feed. They were subjected to standard husbandry practices but not to any additional stressors.

From 1st April 2018, each tank was inspected visually (without catching the fish to minimise stress) on a daily basis to identify females that were gravid and ready to spawn; the readiness to spawn was indicated by an expanded abdomen and dilated anal papilla (Wootton, 1976). Gravid females were removed from the tanks, weighed and measured, lightly anesthetised in 50mg/L benzocaine solution and stripped of eggs. The eggs were photographed on a lightbox before being fertilised *in vitro* with the sperm of a randomly selected sexually mature unrelated male from the stock population. Fertilised eggs were placed in a mesh egg basket suspended within a 10 L plastic tank filled with water from the aquarium system (with approx. 0.5 mL of methylene blue added as a disinfectant) and fitted with an air stone for water oxygenation. The *in vitro* procedure, including selection of males for breeding, was the same as in the maternal population and is described in detail in Chapter 3. Following stripping, the females were placed in temporary holding tanks to avoid breeding more than once from the same female, so that all the results in this experiment are based on the first clutch produced by the F1 females. The inspection of tanks continued daily until up to three females per family had reproduced, or until the end of the breeding season.

Determining clutch characteristics and grandoffspring developmental trajectories

The following parameters were measured and recorded from the reproductive investment of the F1 females:

- Clutch size: from photographs taken at the time of stripping
- Mean mass of single eggs: total clutch mass/number of eggs
- Mean egg volume: using formula volume = $4/3\pi r^3$, with radius (r) determined from photographs using ImageJ processing software (Rasband, 2016)

The following developmental parameters of the F2 offspring were measured:

- Temperature-corrected development time: expressed as Accumulated Thermal Units (ATU; number of days between fertilisation and hatching*average temperature); higher ATU values represent longer development
- Hatching rate: number of hatched fry/number of fertilised eggs
- Mean fry size at hatching: from photographs taken on the day of hatching, measured using ImageJ software

Detailed information on the above procedures is provided in Chapter 3.

Data analysis

All statistical analyses were performed in R statistical software (version 3.4.3, R Development Core Team), with Linear Mixed Models (LMM) fitted using the "lme4" package in R (Bates *et al.*, 2015). The degrees of freedom were estimated by Satterthwaite approximation (for normally distributed data) and Laplace approximation (for Poisson data). P values were obtained from t-statistics or z-statistic using the "lmerTest" package, with an alpha level of 0.05 (Kuznetsova *et al.*, 2017). For all models, non-significant terms were removed by backwards selection. F0 female ID was included as a random factor in all analyses since they used multiple measurements (clutches) from the same family, i.e. from fish that shared the same mother.

A LMM with Poisson distribution was used to analyse the sizes of F2 clutches; these clutches were produced by F1 mothers originating from the three successive breeding attempts of F0 females from the two treatment groups, with a sample size of 80 F2 Control clutches (F1 Clutch 1 n=33, F1 Clutch 2 n=26, F1 Clutch 3 n=21) and 84 F2 Experimental clutches (F1 Clutch 1 n=25, F1 Clutch 2 n=30, F1 Clutch 3 n=29). The fixed factors included in the initial model were F0 treatment group (categorical variable), F1 clutch of origin (either the first, the second or the third clutch produced by an F0 female; categorical variable), F1 female somatic mass (covariate), Julian date of egg stripping of the F1 female (covariate) and the interaction terms F0 treatment group* F1 clutch of origin and F0 treatment group* F1 female mass.

LMMs on the same sample size were used for comparison of single egg mass and egg volume across the treatment groups and clutches. The fixed factors included

in the initial model of single egg mass were F0 treatment group (categorical variable), F1 clutch of origin (categorical variable), F1 female somatic mass (covariate) and the interaction terms F0 treatment group* F1 clutch of origin and F0 treatment group* F1 female mass. The fixed factors included in the initial model of egg volume were F0 treatment group, F1 clutch of origin, single egg mass (covariate) and the interaction terms F0 treatment group* F1 clutch of origin and F0 treatment group*single egg mass.

A LMM was used to analyse the potential differences in the temperature-corrected development time (as ATU) of F2 embryos originating from the two grandmaternal treatment groups. Seven out of 164 *in vitro* crosses did not result in any hatched fry, so that the final sample size for development time analysis was 75 F2 Control families (F1 Clutch 1 n=30, F1 Clutch 2 n=25, F1 Clutch 3 n=20) and 82 F2 Experimental families (F1 Clutch 1 n=23, F1 Clutch 2 n=30, F1 Clutch 3 n=29). The fixed factors included in the initial model of development time were F0 treatment group (categorical variable), F1 clutch of origin (categorical variable), F1 female somatic mass (covariate), single F2 egg mass (covariate), Julian date of F2 egg fertilisation (covariate) and the interaction terms F0 treatment group* F1 clutch of origin and F0 treatment group* F2 single egg mass.

Hatching rate of F2 clutches originating from the two treatment groups was analysed by a LMM with the original sample size: 80 F2 Control and 84 F2 Experimental families. The fixed factors included in the initial model were F0 treatment group (categorical variable), F1 clutch of origin (categorical variable), single F2 egg mass (covariate), as well as the interaction terms F0 treatment group* F1 clutch of origin and F0 treatment group*single F2 egg mass. The response variable (F2 hatching rate) was arcsine-transformed.

A LMM on the reduced sample size of 75 F2 Control and 82 F2 Experimental families was used to analyse the differences in size at hatching of F2 fry. The fixed factors included in the initial model were F0 treatment group (categorical variable), F1 clutch of origin (categorical variable), F2 egg volume (covariate) and F2 development time (as ATU, covariate), as well as the interaction term F0 treatment group* F1 clutch of origin and the interactions between all of the covariates and the F0 treatment group.

Results

Clutch and egg characteristics

The average number of eggs per clutch produced by F1 daughters of stress-exposed mothers did not differ from that of control daughters, but F1 females originating from the middle clutches (Clutch 2) of the F0 generation produced significantly more eggs than those from early (Clutch 1) or late (Clutch 3) clutches (Table 6.1, Figure 6.2). Overall, heavier F1 females produced larger clutches, but there was a significant interaction between F0 treatment group and F1 somatic mass, with heavy F1 females (>1.1 g) from the Experimental group producing smaller clutches relative to the Control females of the same body mass (Table 6.1, Figure 6.3). In addition, the size of clutches produced by the F1 females declined significantly throughout the breeding season (Table 6.1, Figure 6.3).

F1 females produced late in the season (Clutch 3) by Experimental mothers had significantly heavier eggs than the daughters of Control females from the corresponding clutch (Table 6.1, Figure 6.2). Moreover, exposure of F0 females to a period of chronic stress influenced the relationship between the mass of F1 females and the mass of the F2 eggs that they produced: heavy F1 females (>1.1 g) from the Experimental group produced heavier eggs relative to the F1 of the same body mass originating from the Control group (Table 6.1, Figure 6.4). F2 clutches produced later in the breeding season were significantly heavier, regardless of the grandparental treatment (Table 6.1, Figure 6.4). Egg volume did not differ between clutches produced by F1 females originating from the 1st, the 2nd or the 3rd clutch of F0 females (Table 6.1, Figure 6.2). However, the relationship between mass and volume of single eggs produced by F1 mothers was modified by the exposure of the grandmothers to chronic stress. Lighter eggs (< 3 mg) of F1 females whose mothers were from the Experimental group tended to be less dense (i.e. had comparatively lower mass per unit volume) than were the eggs with corresponding mass produced by F1 females whose mothers were from the Control group. This trend reversed for eggs weighing > 3.8 mg, with heavier eggs originating from the Experimental group tending to have higher density than those originating from the Control group (Table 6.1, Figure 6.5). Plotting the volume of single eggs against their mass indicated the presence of a potentially influential observation with a high value for single egg

mass (Figure 6.5). However, this observation did not have a strong influence on model parameters; it had a Cook's distance of < 1 and was less than three standard deviations from the mean. Removal of this observation from the model yielded a result that was qualitatively similar to the original model and thus it was retained in the analysis.

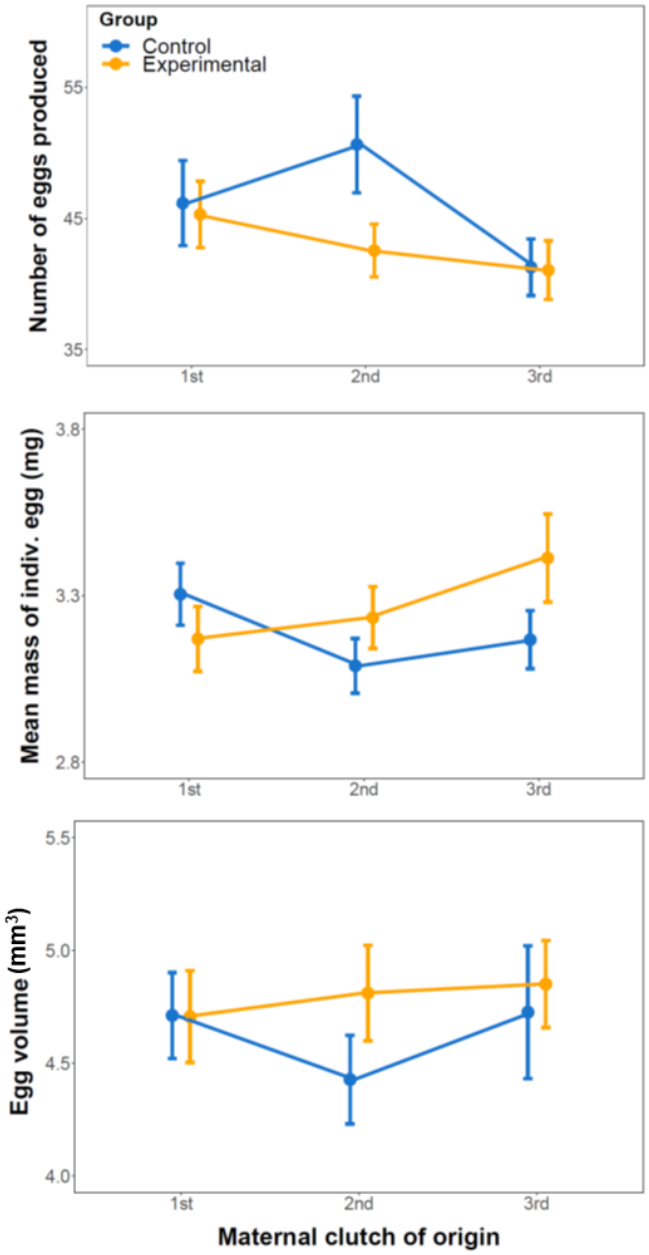

Figure 6.2 The differences in clutch size (as mean number of eggs per clutch, top panel), mean mass of an individual F2 egg (in mg, middle panel) and mean F2 egg volume (in mm^3, bottom panel) of clutches produced by F1 females originating from the 1st, the 2nd or the 3rd clutch of F0 females. The F1 females were not subjected to stressors, but were the offspring of either stress-exposed (orange) or Control (blue) F0 mothers. Sample size: Experimental group n=84 F2 clutches (F1 Clutch 1 n=25, F1 Clutch 2 n=30, F1 Clutch 3 n=29), Control group n=80 F2 clutches (F1 Clutch 1 n=33, F1 Clutch 2 n=26, F1 Clutch 3 n=21). The error bars represent Standard Error of the Mean (s.e).

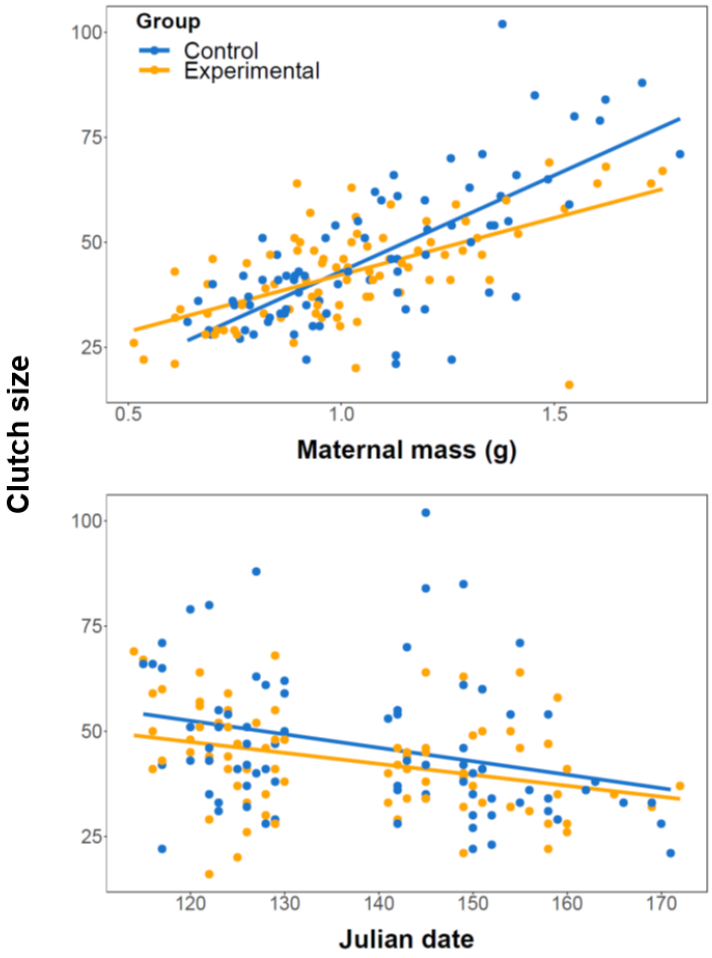

Figure 6.3 The relationship between mass (in grams) of F1 female three-spined sticklebacks and the number of F2 eggs they produced (clutch size; top panel), and between Julian date of F2 egg stripping and F2 clutch size (bottom panel). The F1 females were not subjected to stressors, but were the offspring of either stress-exposed (Experimental, n=84 F2 clutches; orange) or Control (n=80 F2 clutches; blue) F0 mothers. The regression lines were fitted using linear smoothing.

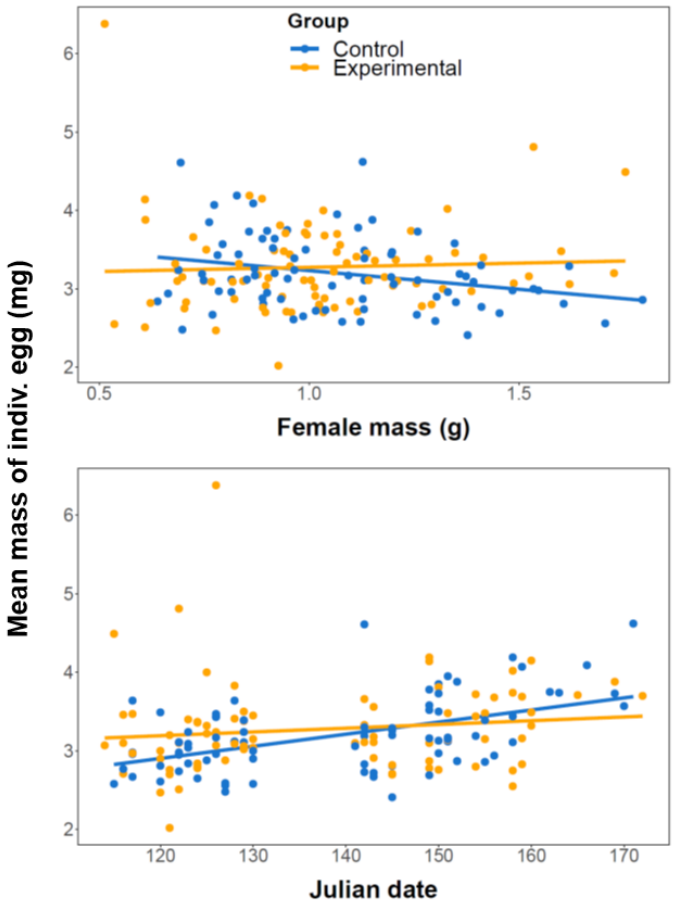

Figure 6.4 The relationship between mass (in grams) of F1 female three-spined sticklebacks and mean mass (in mg) of individual F2 eggs that they produced (top panel), and between Julian date of F2 egg stripping and mean mass of individual F2 eggs (bottom panel). The F1 females were not subjected to stressors, but were the offspring of either stress-exposed (Experimental, n=84 F2 clutches; orange) or Control (n=80 F2 clutches; blue) F0 mothers. The regression lines were fitted using linear smoothing.

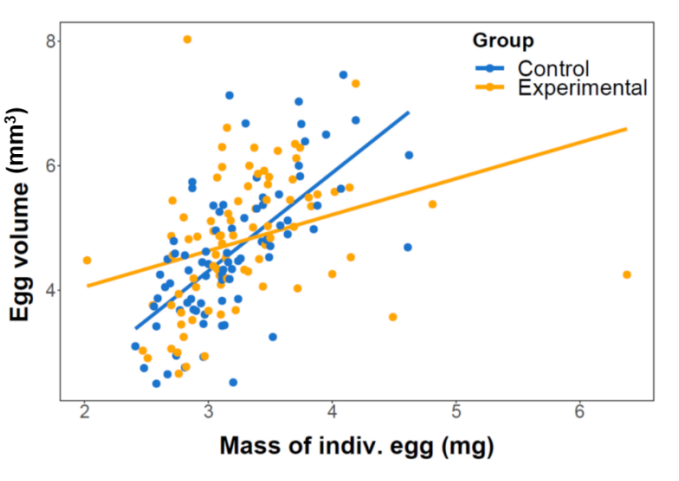

Figure 6.5 The relationship between mean mass (in mg) and volume (in mm^3) of individual F2 eggs produced by F1 female three-spined sticklebacks. These females were not subjected to stressors, but were the offspring of either stress-exposed (Experimental, n=84 F2 clutches; orange) or Control (n=80 F2 clutches; blue) F0 mothers. The regression lines were fitted using linear smoothing. Low values of egg volume for a given egg mass indicate high density.

Table 6.1 Summary of Linear Mixed Models (LMM) used to analyse the differences in F2 clutch size, single F2 egg mass (in mg) and F2 egg volume (in mm^3) produced by non-stressed F1 female three-spined sticklebacks. These F1 females originated from three successive clutches produced by Control (n=80 F2 clutches; F1 Clutch 1 n=33, F1 Clutch 2 n=26, F1 Clutch 3 n=21) and Experimental (n=84 F2 clutches, F1 Clutch 1 n=25, F1 Clutch 2 n=30, F1 Clutch 3 n=29) F0 females. Values give estimates ± s.e. The reference Treatment in all models was the Control group, and the reference clutch was the first clutch in the season. F0 female ID was included in the models as random factor. F0 treatment group, F1 clutch of origin and all significant terms (indicated with p-values in underlined and bold font) were retained in the final models.

Model terms	**Effect on clutch size**	**Effect on egg mass**	**Effect on egg volume**
F0 Treatment group	-0.044 ± 0.042, z=-1.045, p=0.296	-1.044 ± 0.368, $t_{1,120.96}$=-2.833, p=0.005	3.571 ± 0.931, $t_{1,155.19}$=3.834, **p<0.001**
F1 Clutch 2	0.103 ± 0.049, z=2.103, **p=0.035**	-0.200 ± 0.135, $t_{2,53.69}$=-1.485, p=0.143	0.065 ± 0.216, $t_{2,56.52}$=0.302, p=0.764
F1 Clutch 3	0.076 ± 0.053, z=1.443, p=0.149	-0.232 ± 0.150, $t_{2,52.69}$=-1.545, p=0.128	0.091 ± 0.225, $t_{2,54.35}$=0.406, p=0.687
F0 Treatment group* F1 Clutch 2	-0.128 ± 0.097, z=-1.319, p=0.187	0.210 ± 0.196, $t_{2,54.31}$=1.071, p=0.289	-0.172 ± 0.459, $t_{2,52.52}$=-0.373, p=0.711
F0 Treatment group* F1 Clutch 3	-0.093 ± 0.106, z=-0.882, p=0.378	0.446 ± 0.209, $t_{2,52.58}$=2.138, **p=0.037**	0.016 ± 0.026, $t_{2,70.94}$=0.605, p=0.547
F1 Female mass	0.258 ± 0.021, z=12.064, **p<0.001**	-0.519 ± 0.221, $t_{1,139.34}$=-2.350, **p=0.020**	NA
F0 Treatment group* F1 Female mass	-0.095 ± 0.031, z=-3.100, **p<0.001**	0.889 ± 0.306, $t_{1,132.27}$=2.906, **p=0.004**	NA
Julian date	-0.058 ± 0.016, z=-3.690, **p=0.002**	0.010 ± 0.003, $t_{1,140.83}$=3.709, **p<0.001**	NA
Mass of indiv. F2 egg	NA	NA	1.612 ± 0.224, $t_{1,154.84}$=7.200, **p<0.001**
F0 treatment group*mass of indiv. F2 egg	NA	NA	-1.074 ± 0.284, $t_{1,152.82}$=-3.779, **p<0.001**

Development time, hatching rate and fry size at hatching

The temperature-corrected development time (as ATU) of F2 embryos was independent of grandmaternal (F0) treatment and maternal (F1) clutch of origin (Table 6.2, Figure 6.6). Moreover, maternal mass and individual egg mass had no effect on the timing of embryo development (Table 6.2). Likewise, there was no effect of F0 treatment, F1 clutch of origin and F2 egg volume on proportion of F2 eggs that hatched (Table 6.2).

A significant interaction between F0 treatment and F1 clutch of origin indicated that F1 females from clutches spawned later in the breeding season by stress-exposed F0 mothers produced F2 fry that were larger at hatching than corresponding fry from the Control group (Table 6.2, Figure 6.6). The size of F2 fry at hatching was also significantly influenced by the interaction between grandmaternal treatment and their own development time. The size of grandoffspring of the Control females increased with the increased development time, i.e. the fry with higher ATU value were larger at hatching. However, the F2 fry originating from the Experimental grandmothers showed no relationship between these two factors, with size at hatching being relatively constant, regardless of the development time (Table 6.2, Figure 6.7).

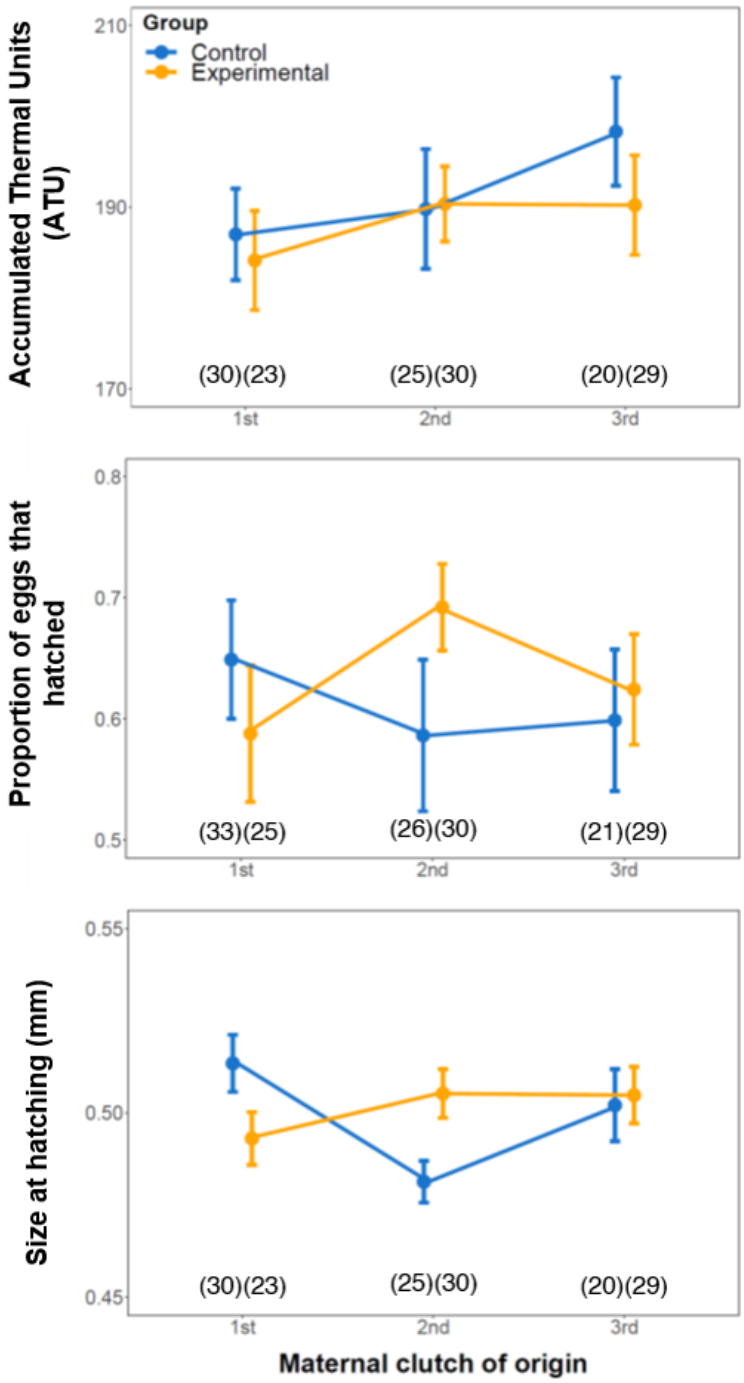

Figure 6.6 The differences in F2 development time (as Accumulated Thermal Units, top panel), hatching proportion (middle panel) and size of F2 fry at hatching (in mm, bottom panel) in offspring of non-stress exposed F1 females. These F1 females originated from three successive clutches produced across the breeding season by F0 female three-spined sticklebacks from Control (blue; n=80 F2 clutches for hatching rate, n=75 F2 clutches for ATU and size at hatching) and Experimental (orange; n=84 F2 clutches for hatching rate, n=82 F2 clutches for ATU and size at hatching) treatment groups. Sample sizes of F2 clutches for each F1 clutch of origin are given in brackets. The error bars represent Standard Error of the Mean (s.e). Note that higher ATU values indicate slower development.

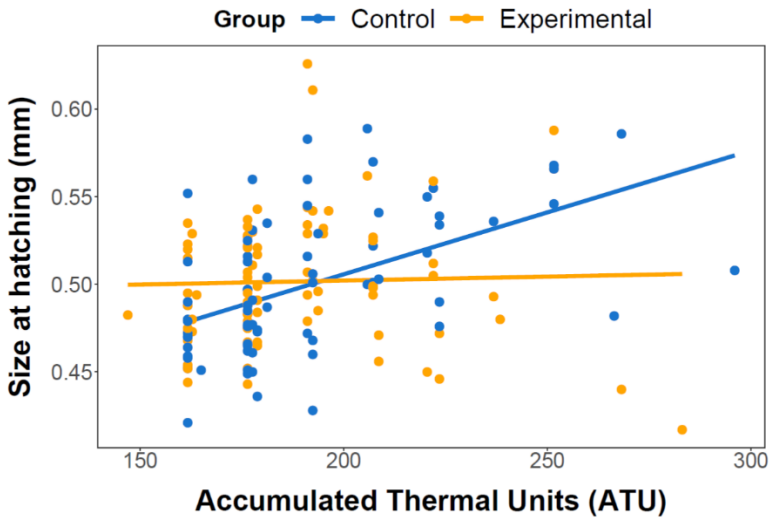

Figure 6.7 The relationship between mean fry size at hatching (in mm) and development time (as Accumulated Thermal Units) of F2 three-spined sticklebacks. Their mothers (F1) were not subjected to stressors, but were the offspring of either stress-exposed (Experimental, n=82 F2 clutches; orange) or Control (n=75 F2 clutches; blue) F0 females. The regression lines were fitted using linear smoothing. Note that higher ATU values indicate slower development.

Table 6.2 Summary of Linear Mixed Models (LMM) used to analyse the differences in F2 development time (as Accumulated Thermal Units), F2 hatching rate and size of F2 fry at hatching (in mm) of offspring produced by non-stressed F1 female three-spined sticklebacks. These F1 females originated from three successive clutches produced by Control (n=80 F2 clutches; F1 Clutch 1 n=33, F1 Clutch 2 n=26, F1 Clutch 3 n=21) and Experimental (n=84 F2 clutches, F1 Clutch 1 n=25, F1 Clutch 2 n=30, F1 Clutch 3 n=29) F0 females. Values give estimates ± s.e. The reference Treatment in all models was the Control group, and the reference clutch was the first clutch in the season. Family ID was included in the models as random factor. Treatment group, Clutch and all significant terms (indicated with p-values in underlined and bold font) were retained in the final models.

Model terms	**Effect on dev. time (ATU)**	**Effect on hatching rate**	**Effect on size at hatching**
F0 Treatment group	-3.548 ± 5.645, $t_{1,47.05}$=-0.629, p=0.533	0.022 ± 0.061, $t_{1,57.65}$=0.358, p=0.722	-0.022 ± 0.011, $t_{1,61.35}$=-2.057, **p=0.044**
F1 Clutch 2	4.724 ± 6.709, $t_{2,48.81}$=0.704, p=0.485	0.022 ± 0.073, $t_{2,59.29}$=0.309, p=0.758	-0.034 ± 0.010, $t_{2,59.47}$=-3.284, **p=0.002**
F1 Clutch 3	6.874 ± 7.018, $t_{2,46.52}$=0.980, p=0.332	-0.023 ± 0.076, $t_{2,56.55}$=-0.306, p=0.761	-0.020 ± 0.011, $t_{2,57.33}$=-1.738, p=0.087
F0 Treatment group* F1 Clutch 2	1.378 ± 13.861, $t_{2,45.98}$=0.099, p=0.921	0.207 ± 0.147, $t_{2,57.43}$=1.403, p=0.166	0.046 ± 0.015, $t_{2,57.83}$=3.080, **p=0.003**
F0 Treatment group* F1 Clutch 3	-2.438 ± 14.527, $t_{2,43.73}$=-0.168, p=0.867	0.134 ± 0.153, $t_{2,54.81}$=0.874, p=0.383	0.031 ± 0.016, $t_{2,55.68}$=2.016, **p=0.049**
F1 Female mass	0.001 ± 0.009, $t_{1,150.86}$=0.135, p=0.892	NA	NA
F2 Egg volume	-1.830 ± 1.992, $t_{1,146.35}$=-0.919, p=0.360	0.014 ± 0.025, $t_{1,157.63}$=0.544, p=0.587	0.004 ± 0.003, $t_{1,145.49}$=1.494, p=0.137
F0 treatment group*F2 egg volume	-0.938 ± 4.053, $t_{1,145.82}$=-0.231, p=0.817	0.051 ± 0.051, $t_{1,152.94}$=-1.005, p=0.317	0.003 ± 0.005, $t_{1,144.21}$=0.644, p=0.520
F2 Development time	NA	NA	0.020 ± 0.004, $t_{1,143.79}$=5.107, **p<0.001**
F0 treatment group*F2 dev. time	NA	NA	-0.020 ± 0.006, $t_{1,142.17}$=-3.418, **p<0.001**

Discussion

This study provides evidence that the reproductive strategy adopted by females can have far-ranging consequences for future generations in terms of their life history and own reproductive strategy, and that this relationship may be further modified by exposure of females to chronic environmental stress. For example, I observed that the clutch size of females was dependent on the clutch size of their mothers, but this relationship was inverse, i.e. females originating from clutches that were significantly smaller (Clutch 2; see Chapter 3 for details) produced larger clutches upon reaching sexual maturity, regardless of maternal treatment. This suggests a trade-off between offspring number and offspring quality adopted by females in the F0 population, which may have had fitness consequences in the next generation. Sticklebacks in this study remained in their family groups after hatching, and the size of the groups was largely dependent on the number of laid eggs; therefore the observed effect on F1 female clutch sizes may also be related to the social conditions experienced by these females. It has been previously shown that the size of a brood in which females are raised and their social environment can have a profound effect on reproductive success in other vertebrates (Naguib *et al.*, 2006, Bian *et al.*, 2015), which could explain the increased fecundity in females originating from smaller clutches.

I also observed a shift in the clutch size–egg mass relationship across the breeding season of the F1 females, with the size of clutches declining but egg mass increasing over time. However, this was independent of the F0 treatment group. Whilst this relationship was modified in the first generation, with exposure to chronically stressful conditions leading to F0 mothers producing both larger clutches and heavier eggs (Chapter 3), grandmaternal stress treatment seems to have lost its importance for these characteristics in F2 clutches. The mass of vertebrate eggs is thought to reflect their overall quality and predict offspring fitness (Williams, 1994, Bobe and Labbe, 2010, Ruuskanen *et al.*, 2011). It is therefore safe to assume that the observed seasonal shift in the F2 clutches results from increased maternal investment in offspring quality towards the end of the reproductive lifespan in this annual fish species, when the trade-off between self-maintenance and reproduction loses its importance (Bell, 1999, Poizat *et al.*, 1999), rather than from any carry-over effects of stressful conditions experienced by the F0 generation.

In this study I have also found a complex relationship between a female's exposure to chronic stress, the body mass of her daughters at sexual maturity and the number and mass of eggs these daughters produced. Across various vertebrate taxa, larger individuals tend to have higher productivity in terms of the number of eggs (in den Bosch and Bout, 1998, Vallin and Nissling, 2000, Pellerin *et al.*, 2016); this was also observed here in F1 females when F0 treatment was not taken into account. Whilst stress-exposed F0 females did not show any alterations of their clutch size cf. the controls (Chapter 3), there was a clear effect of maternal stress on the body mass - clutch size relationship in their daughters, such that heavier F1 individuals that would normally be expected to produce larger clutches were relatively less productive than their counterparts that were the daughters of Control fish. Whilst this is at odds with the theory predicting that additional resources (which heavier females potentially possess) should be invested in increasing individual productivity (Christians, 2002, Einum and Fleming, 2002), it may indicate a delayed effect of stressful environmental conditions manifesting itself in the F1 population. Thus, assuming an anticipatory transgenerational effect, stress exposure in the F0 generation might have pre-programmed F1 females to increase investment in quality rather than quantity of their eggs, as indicated also by the tendency of F1 females originating from stress-exposed mothers to produce eggs with higher density. This however is incongruent with the evidence e.g. from birds (Naguib *et al.*, 2006, Khan *et al.*, 2016a) and reptiles (Sorci and Clobert, 1995), where stressful environmental conditions experienced by mothers did not have an effect on offspring productivity. Whilst certain aspects of individual reproductive strategy may result from the maternal environment, others may require direct exposure to the conditions in question, and some may arise due to the combination of maternal and individual environment (Shama and Wegner, 2014, Mikulski and Pijanowska, 2017); thus to assess the effect of maternal stress on offspring productivity, it may be necessary to put it in the context of the match or mismatch between F0 and F1 environments.

To further add to the complexity of this issue, here I also observed a carry-over effect of chronic maternal (F0) stress on the relationship between F1 female mass and mass of individual F2 eggs, as well as an interaction between the timing of F0 reproduction (i.e. the order of clutches produced by F0 females) and F2 egg mass. Both stress-exposed and control females from the maternal

population (F0) produced heavier eggs later in the breeding season (Chapter 3), which is consistent with the theory of increased investment late in the reproductive lifespan (Poizat *et al.*, 1999). Interestingly, F1 females originating from the clutches produced late in the breeding season themselves produced heavier eggs, but only if their mothers were exposed to chronically stressful conditions. Furthermore, F1 females whose mothers experienced a stressful environment produced significantly heavier eggs for their own mass compared to the daughters of non-stressed females, suggesting increased investment in egg quality in these females. It is possible that individuals that received a greater pre-natal investment are able to invest more resources into their own gametes (Lindström, 1999, Jonsson and Jonsson, 2014); it is however unclear why this effect is only apparent in the offspring of stress-exposed sticklebacks, especially given that egg quality has been previously shown to be lower in offspring of females that experienced unfavourable conditions (Guibert *et al.*, 2013, Langen *et al.*, 2019). The direction of this effect may depend on the severity of the adverse environmental factors, leading either to reduced reproductive investment resulting in suboptimal offspring or to increased investment and offspring adapted to the unfavourable environment (McCormick, 1998, Ennen *et al.*, 2017, Sheriff *et al.*, 2018). I propose that in this study the observed effect may result from interplay between increased maternal provisioning at the end of the breeding season and an anticipatory intergenerational effect, but this hypothesis requires further rigorous testing.

An additional confounding factor at play in this study is the density of single eggs produced by F1 females, which was altered as a result of exposure of F0 females to chronic stress. As expected, there was a positive correlation between volume and mass of eggs, but this was less pronounced in females originating from stress-exposed mothers, which produced eggs that were relatively denser if they were small. According to a recent meta-analysis by Barneche *et al.* (2018), fish egg energy content per unit volume differs between large and small eggs, with the latter having a relatively higher energy content. Therefore, if any intergenerational effects of stress were to be adaptive and to be transmitted to the F2 generation, it would be more beneficial for F1 offspring of stressed mothers to produce eggs that are smaller in size but have a higher energy content and thus be of higher quality overall.

In terms of the development time of F2 embryos, there was no observed grandmaternal effect. The pre-natal development of F1 fish was affected by maternal exposure to chronic stress, with a negative correlation between egg size and embryo development time and a positive relationship between maternal size and embryo development time amongst stress-exposed fish (Chapter 3). However, this effect was not carried over to the next generation, with the development time of F2 embryos being independent of grandmaternal stress treatment; this suggests that a potential effect of chronic stress exposure on developmental trajectories may be short-lived, at least in non-matching environments. Moreover, the development of fish embryos is highly dependent on temperature (Thépot and Jerry, 2015, Pereira *et al.*, 2016, Tsoukali *et al.*, 2016), and it is possible that exposure to stressful conditions may interact with temperature to regulate its rate. Since F1 and F2 embryos developed at different temperatures (12 °C and 15 °C, respectively), the higher developmental temperature of F2 embryos might have offset potential effects of grandmaternal stress. The development time of F2 embryos was also independent of both maternal mass and mass of eggs, which is a surprising finding since in vertebrates both female size (Ronget *et al.*, 2018) and the size of eggs (Self *et al.*, 2018) can have a strong effect on development time.

Regardless of the lack of difference in development time between grandoffspring of stress-exposed and non-exposed females, this study found evidence of a grandmaternal effect on fry size at hatching. In the F1 generation, an increase in size at hatching was observed with each successive clutch produced by females from both treatment groups, suggesting maximisation of maternal investment towards the end of the breeding season (Chapter 3). F1 females originating from late produced clutches gave rise to F2 offspring that were larger at hatching, but only if their grandmother (i.e. the F0 female) was subjected to chronically stressful environmental conditions. In vertebrates larger size at hatching/birth is correlated with increased survival. This has been demonstrated e.g. in birds (Magrath, 1991), reptiles (Nafus *et al.*, 2015) and fish (Garrido *et al.*, 2015), and may provide a survival advantage in stressful conditions (Hopkins *et al.*, 2014). Therefore the observed increase in size at hatching of sticklebacks whose grandmothers experienced stressful conditions may be an example of an anticipatory effect persisting across generations. This has been previously observed in rats (Goetz *et al.*, 2008) and may be true if

stress-exposed females pre-programmed their daughters to invest more in offspring quality, i.e. produce offspring that are larger at hatching (and thus can survive better in the anticipated unpredictable environment), even if F1 female environment does not match that experienced by their mothers (Mikulski and Pijanowska, 2017).

Another argument in favour of the increased investment in offspring quality in response to maternal stress can be found in the relationship between development time and fry size at hatching, with newly hatched F2 sticklebacks originating from stress-exposed grandmothers being relatively large, even if their development time was relatively short. However, accelerated early growth and development can lead to an array of negative effects, including increases in oxidative damage and shortening of telomeres (Alonso-Alvarez *et al.*, 2007, Pauliny *et al.*, 2015, Burraco *et al.*, 2017), which can lead to reduced probability of survival (Nord and Nilsson, 2016), accelerated aging (Ricklefs, 2006) and reduced lifespan (Metcalfe and Monaghan, 2003). Therefore an increased rate of pre-natal growth is only beneficial if the offspring are of high quality and can withstand or offset the negative effects of accelerated growth, or if there are disproportionate advantages to having a large size at hatching.

In summary, in this chapter I provide indirect evidence that the clutch size of female sticklebacks was inversely proportional to the size of clutch from which they originated, and confirm that a shift in the trade-off between productivity and embryo quality can be observed across the breeding season in this annual fish species. I also provide evidence that the effect of chronic environmental stress exposure persists down the maternal line, shaping the reproductive strategy of female offspring of mothers exposed to stress and the early developmental trajectories of the grandoffspring. This effect is present despite the lack of matching of the F0 and F1 environments, i.e. F1 females did not experience the same stressful conditions as their mothers. Exposure of female sticklebacks to a period of chronic stress had an effect on their daughters in terms of clutch size, size of individual eggs and fry size at hatching, and it affected the correlation between volume and mass of individual eggs. I also observed an interaction between the order of maternal clutch, grandmaternal stress treatment and mass of single eggs. The direction of the observed effects suggests that stress-exposed females may pre-program their daughters to produce higher quality offspring; to test this theory it would be beneficial to

follow the later life performance of the F2 generation in both stressful and non-stressful environments, but this was beyond the scope of this study. The observed effects are complex and their adaptive significance is unclear; nonetheless, these results indicate that stressful environmental conditions can have a persistent effect on populations, even if experienced only within a single generation, and that this effect can differ across different reproductive attempts within a generation.

Chapter 7: General discussion

Summary

Environmental factors are an important source of phenotypic variation amongst animals, with the potential to shape various aspects of their morphology, physiology and behaviour. However, various environmental stimuli (e.g. stressors due to unfavourable or unpredictable environmental conditions) not only affect the animals under direct influence of these stimuli, but can also have a profound effect on future generations exerted for example through non-genetic maternal effects (Mousseau and Fox, 1998, Green, 2008). Understanding the mechanisms that drive offspring phenotypic change in response to stressful maternal environment, and the consequences that this change can have for their survival and fitness, has long been a focus of research (Marshall and Uller, 2007, Love *et al.*, 2013, Sheriff *et al.*, 2018). The aim of this thesis was to further this understanding by exploring how exposure to chronically stressful and unpredictable environments affects female reproductive strategy and testing whether potential phenotypic effects of maternal stress differ between the offspring from successive breeding attempts. Figure 1.2 in Chapter 1 presents the overview of the longitudinal experiment and the timeline of this study.

Main findings and their significance

Chronic stress effects in mothers

When studying the effects of maternal stress on offspring phenotype, one of the most crucial steps is to determine whether the chosen method of inducing stress does indeed elicit an organismal response in the mother. Perhaps the most widely used method is quantification of baseline and stress-induced glucocorticoid levels, since these hormones are directly related to primary stress response in vertebrates and can have a direct effect on other physiological functions (Sapolsky *et al.*, 2000, Johnstone *et al.*, 2012, Herman *et al.*, 2016). However, exposure to stressful conditions may also result in other phenotypic alterations, such as changes to various condition indices (Leal *et al.*, 2011, van de Pol *et al.*, 2017, Kleist *et al.*, 2018) and modification of behaviour (Piato *et al.*, 2011, Bliley and Woodley, 2012, Krause *et al.*, 2017). In Chapter 2, I used a range of hormonal and whole-body level indices to assess the effect of a chronically stressful environment on female three-spined sticklebacks.

Firstly, I tested the hypothesis, based on previous evidence (Tsalafouta *et al.*, 2015, Madaro *et al.*, 2015) that stress-exposed fish will show elevation in baseline cortisol levels. I also predicted that acute hormonal response would be attenuated in stress-exposed fish, as previously demonstrated in fish (Jeffrey *et al.*, 2014, Madaro *et al.*, 2015) and other taxa (Rich and Romero, 2005, Crespi and Warne, 2013). However, I did not find evidence in support of either of these hypotheses, with no difference in baseline and stress-induced cortisol levels between stress-exposed and non-exposed sticklebacks. There are many possible interpretations of this result, including habituation to stressful conditions at the physiological level or exhaustion of the stress axis due to protracted stress exposure.

Since the hormonal stress response can be highly context-dependent and there is currently no consensus in the published literature as to the direction and magnitude of chronic stress effects, it may be reasonable to use additional indices when measuring animal response to a chronically stressful environment. For example, in Chapter 2 I employed two behavioural measures (activity and latency to feed) to test the hypothesis that chronic stress exposure alters animal behavioural patterns. Despite the lack of observable stress response in terms of cortisol, stress-exposed sticklebacks showed increased activity, which was particularly pronounced during the presentation of stressors. This is consistent with the behavioural response expected from animals under direct (e.g. during stressor exposure) or short-term stress and may be analogous to their response to acute stress exposure (Krause *et al.*, 2017, Lee *et al.*, 2019). However, when experiencing long-term stressful conditions, it may be more adaptive to conserve energy by lowering activity levels in the absence of direct stressors.

Similarly, in Chapter 2 I observed a shift in feeding behaviour, with stress-exposed sticklebacks becoming faster at acquiring food later in the experimental period. I propose that this shift is associated with the lack of behavioural acclimation to stressful conditions and high energetic cost of mounting a stress response, which together result in fish feeding more efficiently in the resting periods in anticipation of the future stressors. Thus in this study I provide evidence that chronic stress may have a lasting behavioural effect even in the absence of observable physiological effect, which further highlights the importance of considering multiple stress indices when assessing the effects of stressful conditions.

Female reproductive strategies

There is extensive evidence that stress exposure can affect an animal's lifetime fitness (Ketterson and Nolan, 1992, Breuner *et al.*, 2008) and that females experiencing stressful conditions can alter their life history and reproductive strategy in response to stress (Schreck *et al.*, 2001, Ricklefs and Wikelski, 2002, Crespi *et al.*, 2013). It is however unclear whether these alterations reflect the trade-off between reproduction and self-maintenance due to the energetic demands of a stress response, are the effect of maternal strategy to adjust offspring phenotype to the anticipated stressful conditions, or perhaps result from the combination of both (Schreck *et al.*, 2001, Groothuis *et al.*, 2005, Schreck, 2010). Moreover, what emerges from the current literature on this topic is the lack of consensus as to the direction and magnitude of this effect in terms of various clutch and egg characteristics, e.g. clutch size, egg size and embryo developmental trajectories (Chapter 3). This is likely to be caused by the complex relationship between maternal environment (including the nature, intensity and timing of environmental stressors), her physiology and the mechanisms driving the transfer of maternal information into developing eggs or embryos (Sheriff and Love, 2013a, Uller *et al.*, 2013, Sheriff *et al.*, 2018).

I explored this topic in Chapter 3 in an attempt to disentangle the relationship between maternal exposure to a chronically stressful environment and her reproductive strategy across the breeding season. I hypothesised that chronic stress exposure would alter seasonal patterns in clutch and egg characteristics and offspring developmental trajectories. What emerged from my results is that chronic stress does affect a female's reproductive strategy, but not in a straightforward way. Instead, a stressful environment interacted with maternal size in shaping offspring developmental trajectories. In addition, chronic stress exposure shifted the maternal strategy towards increased reproductive investment in terms of clutch and egg size. Contrary to my hypothesis, I found no effect of the interaction between maternal stress exposure and the timing of clutch production on shaping of clutch and egg phenotype or offspring developmental trajectory; even though there were some significant inter-clutch variations, these were independent of maternal stress treatment. Moreover, as opposed to the previous evidence from sticklebacks (Giesing *et al.*, 2011) and other animals (e.g. Saino *et al.*, 2005, Meise *et al.*, 2016, Ensminger *et al.*,

2018), egg cortisol level did not reflect that of a mother and thus any observed patterns are unlikely to be driven by glucocorticoid hormones.

What is therefore the significance of the results presented in Chapter 3? Firstly, they indicate that in annual species the trade-off between reproduction and self-maintenance may be a stronger driving force in shaping reproductive strategies and thus may override the effects of chronic stress exposure. Secondly, my results add to a growing body of evidence that there is more complexity to stress-driven reproductive strategies beyond a simple relationship between glucocorticoids and clutch/egg phenotype. Care must therefore be taken when interpreting the effects of stress on female life history and reproductive strategy, as this relationship may be confounded by other factors that may or may not be related to stress exposure.

Offspring phenotype

In addition to shaping reproductive strategies, maternal exposure to stressful environmental conditions is well documented to be an important source of offspring post-natal phenotypic variation, with an effect on a multitude of traits. For example, offspring growth, survival, stress physiology and behavioural phenotype can all be altered by maternal stress exposure, and many of these alterations seem to be driven by maternal hormones (Dufty *et al.*, 2002, Green, 2008, Love *et al.*, 2013, Sheriff *et al.*, 2018). The overarching question is whether these alterations are due to active maternal programming of offspring phenotype, where females attempt to produce offspring with certain phenotypic traits that would allow them to better survive in anticipated stressful environments, or whether they are merely a reflection of maternal environment with no adaptive value for the offspring (Heath and Blouw, 1998, Uller *et al.*, 2013).

In this thesis, I addressed this question by studying the effects of simulated chronic maternal stress on various aspects of offspring phenotype. In Chapter 4, I examined how maternal stress exposure interacts with the timing of reproduction in shaping of offspring post-natal growth and survival. Whilst I found no effect of maternal stress on offspring growth rate, an inter-clutch difference in growth pattern was evident, with late-produced offspring growing fast early on, but showing reduced growth rate and lower body mass later. Why would these late-produced offspring show such fluctuations in growth rate? The

answer may be an increased maternal investment to allow them to reach larger size before the onset of winter; this would be consistent with the observations from Chapter 3, which showed an increase in egg size in clutches produced later in the breeding season. However, rapid early growth often comes at a physiological cost (Metcalfe and Monaghan, 2001, Lee *et al.*, 2012), which could explain a reduced growth rate later on. As with the female reproductive strategy, the observed inter-clutch difference in growth rate may be an example of a situation in which seasonal maternal effects play a more important role than maternal stress effects in shaping offspring phenotypes.

Nonetheless, seasonal and stress-induced maternal effects are not always mutually exclusive. For example, in Chapter 4 I present evidence that exposure of mothers to chronically stressful environment interacts with the timing of reproduction and results in differential patterns of offspring survival. The shape of this relationship suggests that stress-exposed females positively influence the survival of offspring from late produced clutches, but only in the early stages of life, with an increased mortality of these offspring later on. Could a non-matching maternal and offspring environment explain this increase in mortality past the initial juvenile stage? This would be a feasible explanation in light of some current empirical and theoretical evidence (Hoyle and Ezard, 2012, Shama, 2015, Thayer *et al.*, 2018). However, in a recent meta-analysis Yin *et al.* (2019) provide an argument that in vertebrates maternal effects are generally more adaptive in benign (and thus non-stressful) conditions, which is at odds with my hypothesis of increased mortality due to non-matching conditions.

Whilst seasonal maternal adjustments to offspring phenotype are well documented (e.g. You *et al.*, 2009, Maddams and McCormick, 2012, Mitchell *et al.*, 2018), the evidence that these can be modified by maternal stress exposure lasting throughout the entire breeding season is lacking. Therefore Chapter 4 provides a novel insight into the combined effects of chronic stress and seasonality of reproduction on offspring survival. Moreover, in the earlier chapters I showed that chronic stress exposure had no effect on maternal (Chapter 2) and egg (Chapter 3) cortisol levels. Thus the observed effects are unlikely to be driven by cortisol, which highlights the importance of considering mechanisms of maternal adjustments of offspring phenotype that are independent of glucocorticoid hormones.

Glucocorticoid hormones have also been thought to be a link between a stressful maternal environment and offspring behavioural phenotype (e.g. Øverli *et al.*, 2002, Thaker *et al.*, 2009, Piato *et al.*, 2011, Favreau-Peigne *et al.*, 2014). Interestingly, despite the fact that stress-exposed females did not deposit more cortisol in their eggs, in Chapter 5 I observed a marked effect of chronic maternal stress on offspring behaviour, with juveniles produced by stress-exposed females having reduced consistency of behaviour between full- and half-siblings. Since various aspects of animal behaviour are linked with survival (White *et al.*, 2013, Morales *et al.*, 2018), if the environment is unpredictable (and thus it may be more problematic for a mother to predict future environmental conditions) it may be adaptive in terms of offspring survival to produce individuals that vary in their behavioural traits. Thus the results presented in Chapter 5 indicate that exposure to chronically stressful and unpredictable conditions may result in adaptive maternal programming of offspring behavioural phenotypes. I also provide further evidence that glucocorticoids may not be as ubiquitous in translating maternal experience into offspring phenotype as previously thought, with other factors likely to be involved in this process. Moreover, here I observed no direct effect of chronic maternal stress on offspring behaviour but a clear within-family diversification of behavioural traits. It indicates that maternal effects on offspring behaviour are complex in nature and thus it may be critical to consider the same behaviours from various angles when examining the effects of maternal stress.

In addition, to the best of my knowledge the present study is the first one to consider the differences in behaviour of offspring from successive clutches produced by annual fish across their reproductive lifespan. Whilst the variation was small and observed only in terms of sheltering behaviour, these results provide a paradigm for future studies on seasonal variations in maternal programming of offspring phenotype, also in the context of maternal stress.

In Chapter 5 I also examined the effect of chronic maternal stress on offspring response to an acute stressor. Interestingly, but quite unexpectedly in light of the current state of knowledge (e.g. Rich and Romero, 2005, Mommer and Bell, 2013, Weber *et al.*, 2018), offspring of females exposed to an unpredictable environment had neither heightened nor attenuated stress response overall. It is therefore possible that, unlike other phenotypic traits in this study, development of the stickleback HPI axis and subsequent responsiveness to

stressors or novel environmental conditions is highly reliant on maternally-derived glucocorticoids. In this scenario, since there was no detectable effect of chronic maternal stress exposure on female or egg cortisol levels, it would be reasonable to expect a lack of effect on offspring acute stress response. However, it does not explain the modifying effect of maternal stress treatment on inter-clutch differences in offspring acute stress response observed in Chapter 5. In this chapter, I provide suggestive evidence that stress-exposed females influence the stress physiology of their offspring, who show attenuated response to an acute stressor. However, this is only true for the offspring produced late in the breeding season, so this response is a function of both maternal experience and timing of reproduction.

It is important to emphasise that these late-produced offspring of stress-exposed mothers also showed a higher growth rate during early post-natal development. Activation of the vertebrate HPA/HPI axis and subsequent biosynthesis of hormones are energetically expensive (Romero, 2004, Picard *et al.*, 2018) and animals have finite energy resources to allocate to competing functions, e.g. somatic growth and mounting of a stress response (Moore and Hopkins, 2009, Cornelius *et al.*, 2011, Mogensen and Post, 2012). Offspring produced late in the breeding season have limited time to reach a size that improves their chances of surviving winter; therefore if stress-reactivity comes at a cost of a reduced growth rate (and thus increased risk of overwinter mortality due to small size), it may be adaptive for females to produce offspring with attenuated stress responses but faster growth rate. Why would this only be apparent in stress-exposed females? Small body size and adversity of environmental conditions may act synergistically at increasing the risk of overwinter mortality (Shoup and Wahl, 2011, Anderson and Scharf, 2014). Therefore if a female anticipates stressful future conditions, it may be adaptive for her to adjust the phenotype of her offspring to offset the trade-off between physiological stress response and growth.

Effects further down the maternal line

Evidence from this and other studies shows that protracted exposure to unpredictable environmental conditions can have far-ranging consequences for female reproductive strategies and various aspects of the offspring pre- and post-natal phenotype (Green, 2008, Crespi *et al.*, 2013, Sheriff and Love,

2013b). But can the effects of chronic stress exposure be carried over to subsequent generations? I addressed this question in Chapter 6, where I examined effects of chronic maternal stress on offspring reproductive decisions and grandoffspring pre-natal phenotype, with emphasis on differences between offspring from successive breeding attempts. I demonstrated in earlier chapters that many of these inter-clutch differences were modified by maternal (F0) exposure to stressful conditions; I thus predicted that seasonal maternal adjustments due to chronic stress exposure could have implications for future generations, for example by affecting reproductive decisions of F1 offspring derived from different breeding attempts.

Not all traits under scrutiny in Chapter 6 were affected by exposure of F0 females to the chronic stress protocol. For example, the development time of F2 embryos was independent of grandmaternal treatment. Therefore, even though changes in development time were observed in F1 embryos whose mothers were stress-exposed (Chapter 3) this effect was not carried over to the next generation. However, many of the F1 female reproductive traits showed a complex relationship with F0 treatment in a manner suggesting that stress-exposed females might have pre-programmed their daughters to invest more in the quality of their progeny. For example, F1 females spawned by stress-exposed mothers tended to produce eggs that were heavier and denser, and thus potentially of higher quality (Barneche *et al.*, 2018); nonetheless, this relationship was not straightforward and depended on factors such as female size.

Perhaps the most convincing argument in favour of anticipatory maternal programming persisting down the female line stems from the way in which an interaction between F0 treatment and timing of reproduction affected mass of the eggs produced by their daughters and size at hatching of their grandoffspring. Late-spawned F1 daughters of stress-exposed females had higher early life growth rates (Chapter 4) and lower responses to an acute stressor (Chapter 5), suggesting increased maternal investment. These late-spawned sticklebacks themselves produced eggs that were heavier, and fry hatched from these eggs were larger at hatching, even if their development time was short. Thus in Chapter 6 I demonstrate that increased maternal investment due to a combination of seasonality and unpredictability of the environment can have

consequences for future generations if it influences reproductive investment of their daughters.

What emerges from these results is that the relationships between various maternal and egg/offspring characteristics are complex and that their complexity may increase across generations, for example due to added confounding factors resulting from non-matching maternal and offspring environments. Moreover, some effects of exposure to chronically stressful environments may be unclear in the first generation but may become more explicit in later generations, and they can interact with seasonal patterns in reproductive investment to have a different effect on offspring from successive breeding attempts. Therefore, when assessing the implications of chronic environmental stress, it may be important to look not only at multiple generations, but also at multiple cohorts produced within a generation, as examining one reproductive attempt appears to only provide a snapshot of the processes resulting from maternal stress exposure.

Justification of methodology and study limitations

This study examined transgenerational effects of exposure to stressful and unpredictable environment in wild-caught three-spined sticklebacks. The use of wild individuals in a laboratory setting allowed me to control for potentially confounding environmental parameters (e.g. fluctuations in temperature, food abundance and predation) whilst providing reliable information on behavioural and physiological parameters that are likely to reflect those occurring in the wild. However it has previously been shown that wild and captive vertebrate populations may differ in physiological stress response (Woodward and Strange, 1987, Coburn *et al.*, 2010) and behaviour (Gilby *et al.*, 2013, Salvanes, 2017); hence care must be taken when interpreting the results of this study and extrapolating them to captive animals.

In Chapter 2, I used a scan-sampling approach to analyse female behaviour. This method of behavioural observation has been used in a range of vertebrate taxa, including fish (e.g. Berejikian *et al.*, 2000, Hutter *et al.*, 2010), and it is cost-effective, easy to use and allows to observe multiple individuals at once (Altmann, 1974). Some shortcomings of this observational approach, as compared to automated tracking of behaviour, include lower sampling rate and higher risk of observer bias (Dell *et al.*, 2014). However, since the aim of this

study was to examine the effects of chronic maternal stress, any additional handling of the fish (such as that associated with moving fish to an automated tracking setup) was kept to a minimum to reduce the risk of eliciting acute stress response. Because automated tracking was used to analyse offspring behaviour in Chapter 5, it was impossible to determine whether offspring behavioural traits reflect those of a mother, which limits the conclusions that can be made from this study. Whilst in the present study this question was of secondary importance, future studies should carefully consider pros and cons of each observation method taking into account main questions and hypotheses.

In Chapters 2 and 5, I measured water-borne cortisol to determine baseline and peak cortisol levels in females and peak cortisol levels in offspring, respectively. This method has been used in a wide range of fish species and it has a clear advantage over traditional methods (e.g. plasma cortisol) which, depending on fish size, are either invasive or destructive. Extraction and quantification of water-borne steroids is non-destructive and non-invasive, minimally stressful and allows multiple measurements from the same individual (Scott *et al.*, 2008, Sadoul and Geffroy, 2019), which is particularly important in small fish and in longitudinal studies, such as that presented in Chapter 2. Water-borne cortisol levels have also been shown to be a reliable estimate of the plasma cortisol levels in the fish living in that water (Ellis *et al.*, 2004, Gabor and Contreras, 2012, Félix *et al.*, 2013), and due to a time lag between cortisol production in interrenal cells and the diffusion of hormones into water it is relatively easy to measure both baseline and peak levels (Sebire *et al.*, 2009, Pavlidis *et al.*, 2013). It is generally recommended to validate the relationship between water-borne and plasma cortisol prior to each study (Scott and Ellis, 2007, Sebire *et al.*, 2007), but due to problems with obtaining plasma samples from sticklebacks, I was unable to perform this validation. However, measurement of water-borne cortisol in three-spined sticklebacks has been previously validated (Sebire *et al.*, 2007, Sebire *et al.*, 2009) and thus I was confident in using this method without additional validation.

In Chapter 3, I measured cortisol in non-fertilised stickleback eggs using whole egg homogenate without extraction. Whilst traditionally cortisol from eggs is extracted prior to quantification (Mileva *et al.*, 2011, Sopinka *et al.*, 2014, Taylor *et al.*, 2016), there is increasing evidence that cortisol values in extracts do not differ from those obtained from egg homogenates (Giesing *et al.*, 2011,

Faught *et al.*, 2016). In light of this evidence, I chose to use the latter as a time- and cost-effective alternative to extraction. However, my results of egg cortisol levels should be treated with a degree of caution due to the small sample size. One of the reasons was the small size of some clutches; since offspring developmental and phenotypic changes due to maternal stress exposure may be more informative in this context, it was deemed inappropriate to sacrifice the eggs for cortisol analysis if it left few or no eggs for fertilisation.

Also in Chapter 3, the aim was to continue collecting clutches throughout the breeding season to allow for comparison between all clutches produced by females from an annual population throughout their reproductive lifespan. However, most females in this study (both stress-exposed and non-exposed) ceased spawning after producing two or three clutches; this is unexpected since wild sticklebacks can produce as many as nine clutches in a single breeding season (Whoriskey and FitzGerald, 1994, Baker *et al.*, 2015). It is unclear why the reproductive output observed in Chapter 3 was low regardless of whether the fish were exposed to stressors or not; nonetheless, to allow for comparison and to avoid potential confounding factors due to large differences in the number of breeding attempts, the breeding was capped at three clutches.

A number of limitations due to time and financial constraints can be observed in Chapter 5, where I examined aspects of offspring stress physiology and behaviour in response to chronic maternal stress. Firstly, I only measured peak cortisol level following exposure to an acute stressor, as maternal stress has previously been shown to lead to altered response to stressors (e.g. Hayward and Wingfield, 2004, Redfern *et al.*, 2017, Weber *et al.*, 2018). However, where comparisons are made between cortisol levels and behavioural traits, baseline levels may be superior to peak levels at predicting behaviour (Silva *et al.*, 2010). Secondly, for the purpose of cortisol quantification I pooled water samples from all sampled individuals in each family (three fish per family) to obtain a mean value per family. This approach however may lead to loss of information if the inter-individual differences in hormonal response are large. In addition, cortisol synthesis may differ between individuals of different age or life stage (Koakoski *et al.*, 2012, Tsalafouta *et al.*, 2015), and the differences can be seen in terms of time-course of release as well as in the average cortisol level (Mommer and Bell, 2013, Tilgar *et al.*, 2016). It would therefore be recommended to measure cortisol in offspring of different ages, starting shortly after fertilisation to

account for potential embryonic regulation of maternally-derived hormones (Paitz *et al.*, 2015), and at different time points after the exposure to an acute stressor, but this was not possible in the current study due to the high cost of water-borne cortisol quantification.

Potential future avenues of research

Perhaps the biggest challenge facing researchers of maternal stress effects is the inability to measure glucocorticoid hormones in a way that is completely non-invasive and does not itself elicit stress. In addition, glucocorticoids in teleost fish and other vertebrate taxa show a complex pattern of biosynthesis, metabolic clearance and uptake, and this pattern can change on a diurnal and more long-term scale (Mommsen *et al.*, 1999, Dickmeis, 2009); thus to fully disentangle the effects of stress exposure from these natural fluctuations, it would be beneficial to use methods allowing for continuous monitoring of cortisol levels without disturbing the fish. Recent years have seen the emergence of methods with the potential to be used in this context, such as electrochemical or piezoelectrical biosensing and the use of aptamers (antibody alternatives; reviewed by Sadoul and Geffroy, 2019); however, these methods are yet to be validated for continuous quantification of water-borne cortisol. I would therefore argue that establishment of a robust method of measuring low concentrations of cortisol in holding water should be the most important next step in the study of stress effects in fish.

Furthermore, as indicated by the results of Chapter 2, exposure to stressful environmental conditions can have an impact on behaviour even in the apparent absence of hormonal stress response. Future studies are therefore required that aim to better establish the link between protracted stress exposure and various aspects of behaviour, and how these behaviours change over time if the stressful conditions persist. Recent technological advances in the field of automated animal tracking allow for tracking of complex behaviours in groups of animals (and thus in a more natural setting in shoaling fish species such as three-spined stickleback (Grobis *et al.*, 2013)) in a continuous manner (Qian *et al.*, 2016, Itskovits *et al.*, 2017). Use of these advanced methods would not only improve efficiency and accuracy of behavioural observations, but would also allow for novel insights into behavioural effects of chronic stress.

The same tracking methods could be used to build upon the results of Chapter 5, where I demonstrated that exposure of a mother to chronically stressful environment resulted in diversification in the behaviour of her offspring, as indicated by high within-family variability in behavioural traits such as activity levels and use of a shelter. This suggests that females may adopt an adaptive bet-hedging strategy to increase the variability of offspring behaviour in an unpredictable environment. However, maternal influences have been shown to affect offspring personalities (Reddon, 2012), but there has been little research on animal personalities in the context of maternal stress (Groothuis and Trillmich, 2011). Therefore improving the methods used in Chapter 5 and expanding them by performing repeated behavioural measurements of the same individuals would provide a link between chronic maternal stress, within-family variability and individual personality.

Lastly, an underlying theme observed throughout this thesis is the interaction between maternal stress exposure and the order of clutches produced across the breeding season, with factors such as offspring growth rate, survival (Chapter 4) and stress response (Chapter 5) being influenced by this interaction. Moreover, some effects of this interaction were carried over to the subsequent generation (Chapter 6). However, if we are to reliably assess whether the effects of chronic maternal stress accumulate or diminish across a sequence of clutches, it may be of utmost importance for future studies to disentangle the effects of a chronically stressful and unpredictable maternal environment from the effects of seasonal patterns in maternal reproductive investment. The latter is particularly pronounced in annual populations, such as the three-spined stickleback population used in this study. In such populations, mothers can differentially allocate resources between offspring from successive breeding attempts, with an increase in maternal investment towards the end of the breeding season (i.e. the end of their reproductive lifespan), when the trade-off between self-maintenance and reproductive investment loses its importance (Poizat *et al.*, 1999, Duffield *et al.*, 2017).

Therefore future studies may require a shift towards perennial species, where such seasonal patterns may be less pronounced, especially in the first reproductive season (Billman and Belk, 2014). Tropical fish may be a promising system to study transgenerational and inter-clutch effects of maternal stress due to their relatively long lifespan and thus potential for multiple reproductive

seasons (Meekan *et al.*, 2001), and the possibility of inducing reproduction in spawning age females at any time of year by manipulating environmental conditions (Ward and Wyman, 1977, Doherty, 1983). McCormick (1999) demonstrated inter-clutch differences in the size at hatching of Ambon damselfish induced by exogenous cortisol manipulations applied to the mother, whilst Mileva *et al.* (2011) observed that eggs of daffodil cichlids exposed to a repeated acute stressor showed inter-clutch variation in size; these species may thus provide alternative to three-spined sticklebacks for examining various aspects of maternal stress effects.

Alternatively, similar studies can be performed in passerine birds. Within-clutch variation in egg hormones and offspring phenotype, driven by non-genetic maternal effects, has been previously demonstrated in passerines (Groothuis *et al.*, 2005, Love *et al.*, 2008); it is therefore possible that similar differentiation may be observed between clutches if stressful conditions persist throughout the breeding season. Zebra finches have a great potential in this context: these opportunistic breeders reproduce readily in the laboratory, produce multiple clutches in their life time and breeding season can be induced through manipulation of environmental conditions (Perfito, 2010). Moreover, non-genetic maternal influences on offspring phenotype in zebra finches are widely studied and well documented (reviewed by Griffith and Buchanan, 2010), which may aid the interpretation of potential inter-clutch differences. It must however be noted that, unlike three-spined sticklebacks and many other fish species, passerine birds do not produce their eggs synchronously and offspring phenotype can be influenced by the position of the egg in the laying sequence (Rutkowska and Cichon, 2005, Rubolini *et al.*, 2011, Mainwaring and Hartley, 2013). Therefore it may be important to take these intra-clutch differences into account as they may confound the observed relationships between stressful maternal environment and inter-clutch differences in offspring phenotype.

Appendix 1: Index of scientific names

Common name	Scientific name
Ambon damselfish	*Pomacentrus amboiensis*
American red squirrel	*Tamiasciurus hudsonicus*
Antarctic fur seal	*Arctocephalus gazelle*
Asian sea bass	*Lates calcarifer*
Aspic viper	*Vipera aspis*
Atlantic cod	*Gadus morhua*
Atlantic croaker	*Micropogonias undulates*
Atlantic halibut	*Hippoglossus hippoglossus*
Atlantic salmon	*Salmo salar*
Barn swallow	*Hirundo rustica*
Brook trout	*Salvelinus fontinalis*
Brown anole	*Anolis sagrei*
Brown trout	*Salmo trutta*
Chinook salmon	*Oncorhynchus tshawytsha*
Coho salmon	*Oncorhynchus kisutch*
Convict cichlid	*Amatitlania nigrofasciata*
Daffodil cichlid	*Neolamprologus pulcher*
Eastern fence lizard	*Sceloporus undulates*
Eastern mosquitofish	*Gambusia holbrooki*
European perch	*Perca fluviatilis*
European sea bass	*Dicentrarchus labrax*
European starling	*Sturnus vulgaris*
European sturgeon	*Huso huso*

Common name	Scientific name
Gilthead sea bream	*Sparus aurata*
Goldfish	*Carassius auratus*
Great tit	*Parus major*
Guinea pig	*Cavia porcellus*
Haddock	*Melanogrammus aeglefinus*
Japanese quail	*Coturnix coturnix japonica*
Largemouth bass	*Micropterus salmoides*
Mallee dragon	*Ctenophorus fordi*
Pink salmon	*Oncorhynchus gorbusha*
Pygmy perch	*Nanoperca australis*
Rainbow trout	*Oncorhychus mykiss*
Rohu	*Labeo rohita*
Slimy sculpin	*Cottus cognatus*
Sockeye salmon	*Oncorhynchus nerka*
Southern fiddler ray	*Trygonorrhina dumerilii*
Three-spined stickleback	*Gasterosteus aculeatus*
Viviparous lizard	*Zootoca vivipara*
Yellow-legged gull	*Larus michahellis*
Zebra finch	*Taeniopygia gutatta*
Zebrafish	*Danio rerio*

Appendix 2: Female condition factor prior to outlier removal

Table A2.1 Summary of Linear Mixed Models (LMM) used to analyse the effect of Treatment group and the number of clutches produced over the breeding season on female body condition, expressed as Condition Factor (CF). Values give estimate ± s.e. The reference Treatment was the Control group. Female ID was included in the models as a random factor. Control group n=28 females, Experimental group n=29. Number of clutches was used as a factor in the model for final (after UCSP) but not initial (before UCSP) CF. Treatment group and any significant terms (indicated with p-values in underlined and bold font) were retained in the final model. CF before UCSP shows the original analysis prior to the removal of the single abnormally high value for the Control group.

Model terms	CF before UCSP	CF after UCSP
Treatment group	-0.016 ± 0.060, $t_{1,36.19}$=-0.265, p=0.793	-0.043 ± 0.026, $t_{1,55.00}$=-1.644, p=0.106
No. of clutches	n/a	-0.005 ± 0.011, $t_{1,54.00}$=-0.512, p=0.611
Treatment group*No. of clutches	n/a	-0.001 ± 0.021, $t_{1,52.94}$=-0.071, p=0.944

Appendix 3: F0 clutch size prior to outlier removal

Table A3.1 Summary of Linear Mixed Model (LMM) used to analyse the difference in clutch size, in three successive clutches produced by Control and Experimental female three-spined sticklebacks during the breeding season. Values give estimates ± s.e. The reference Treatment in all models was the Control group. Female ID and tank ID were included in the models as random factors. Control group n=20 females (Clutch 1 n=19, Clutch 2 n=20, Clutch 3 n=15), Experimental group n=19 (Clutch 1 n=19, Clutch 2 n=19, Clutch 3 n=14). Treatment group, Clutch and all significant terms (indicated with p-values in underlined and bold font) were retained in the final models. Table shows the original analysis prior to the removal of four clutches with abnormal residuals.

Model terms	Effect on clutch size			
	Est.	s.e.	z value	p value
Treatment group	0.003	0.082	0.034	0.973
Clutch 2	-0.135	0.063	-2.147	**0.032**
Clutch 3	-0.065	0.063	-0.942	0.346
Treatment group*Clutch 2	-0.072	0.126	-0.570	0.569
Treatment group*Clutch 3	-0.049	0.137	-0.357	0.721
Female mass	0.210	0.038	5.534	**<0.001**

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