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Unpredictability of maternal environment shapes offspring behaviour without affecting stress-induced cortisol in an annual vertebrate

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ABSTRACT

Exposure of females to stressful conditions during pregnancy or oogenesis has a profound effect on the phenotype of their offspring. For example, offspring 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 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, particularly in the context of stress lasting throughout entire reproductive lifespan. To bridge this knowledge gap, we exposed female sticklebacks to stressful and unpredictable environmental conditions throughout the breeding season. We quantified the activity, sheltering and anxiety-like behaviour of offspring from three successive clutches of these females, and calculated Intra-class Correlation Coefficients for these behaviours in siblings and half-siblings. We also exposed offspring to an acute stressor and measured their peak cortisol levels. An unpredictable maternal environment had no modifying effect on inter-clutch acute stress responsivity, but resulted in diversification of offspring behaviour, indicated by an increased between-individual variability within families. This may represent a bet-hedging strategy, whereby females produce offspring differing in behavioural phenotype, to increase the chance that some of these offspring will be better at coping with the anticipated conditions.

1. Introduction

Offspring phenotypic change in response to unpredictable maternal environment, and the consequences that this change can have for their survival and fitness, may be of profound importance to persistence of wild populations. This is particularly important considering the ongoing ecological and climate changes, which re-shape the environment and are a source of unpredictability in the wild (Love et al., 2013; Shama, 2015; Sheriff et al., 2018). Notably, in annual organisms (i.e., those that reproduce over a single breeding season), environmental unpredictability and its effects on offspring phenotype may be critical for lifetime reproductive success (Sheriff and Love, 2013), particularly when maternal environmental conditions interact with seasonal changes in maternal allocation. Some of the aspects of a phenotype of an animal that can be influenced by the maternal environment, and that can have a significant effect on its fitness, include various aspects of behaviour as well as the way in which an animal responds to challenges (e.g., stressors) on both physiological and behavioural levels (Ensminger et al., 2018; Redfern et al., 2017; Weber et al., 2018).

Within-species variation in the behaviour of animals tends to be tightly linked with their physiology. This includes the levels of steroid hormones (e.g. glucocorticoids (GCs)), both as a baseline and following stimulation of the hypothalamic-pituitary-adrenal (HPA) axis (or the equivalent hypothalamic-pituitary-interrenal, HPI, axis in fish) by a stressful stimulus (Lemonnier et al., 2022; Romero, 2004). A correlation between hormones (at both baseline and stress-induced levels) and behavioural traits has been demonstrated across a wide range of species and contexts (Garland et al., 2016; Hau and Goymann, 2015; McGlothlin and Ketterson, 2008), including in the context of elevated GC levels following exposure to stressors leading to behavioural changes (Lemonnier et al., 2022; Lupien et al., 2009; Ralph and Tilbrook, 2016). The link between stressor exposure, animal behaviour and stress physiology is often related to alteration of GC signalling pathways (Kleist et al., 2018), change in responsiveness of the stress axis to subsequent stressors

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(Rich and Romero, 2005; Vitousek et al., 2022) and differential expression of genes encoding corticosteroid receptors (Lee et al., 2019; Jimeno and Zimmer, 2022). 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 (Garland et al., 2016; McGlothlin and Ketterson, 2008).

Moreover, if a female experiences stressful conditions during the period of egg production or embryo development, this can have a profound effect on the behaviour and stress physiology of her offspring. This occurs either through direct deposition of maternally derived GCs (or placental transfer of GCs in the case of mammals), where GC levels of eggs/embryos reflect those of a mother (Ensminger et al., 2018; Kleppe et al., 2013; Okuliarová et al., 2010; Sheriff et al., 2017), or through other routes such as mRNAs deposited by mothers in eggs (Colson et al., 2019; Perez and Lehner, 2019). Maternally transferred GCs and mRNAs 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 (Best et al., 2017; Kleist et al., 2018; MacLeod et al., 2021). 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-predator behaviour (Hellmann et al., 2020; Morales et al., 2018), cognitive ability (Eaton et al., 2015; Munch et al., 2018), aggressiveness (Tamilselvan and Sloman, 2017) and boldness (Best et al., 2017; Sopinka et al., 2015).

Similarly, pre-natal exposure to elevated levels of stress hormones (either through maternal stress exposure or exogenous GCs) may lead to altered expression of genes involved in HPA/HPI axis development, resulting in variation of baseline GC levels and altered response to stressful stimuli (Dufty et al., 2002; Love et al., 2013; Romero, 2004). 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 alternatively elevated (Bian et al., 2015; Emack et al., 2008; Strzelewicz et al., 2021) and reduced (Kleist et al., 2018; Mateo, 2014; Tilgar et al., 2016) in response to maternal stress. Hormonal response to a stressor (measured as a peak cortisol level following exposure to an acute stressor) of animals pre-natally exposed to either maternal stress or exogenous stress hormones has also been shown to be suppressed (Jeffrey and Gilmour, 2016; Redfern et al., 2017) or elevated (Cirulli et al., 2009; Soares-Cunha et al., 2018). 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 (Weber et al., 2018) and the regulation of stress responses via negative feedback on the HPA axis (Uehling et al., 2020; Zimmer et al., 2019) may nonetheless be altered.

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 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, such that it is possible for her to anticipate it (Sheriff et al., 2017). 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 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; Sheriff et al., 2017). In such cases, it may be adaptive to adopt a bet-hedging strategy, whereby the offspring differ in their behavioural phenotype, giving at least some of the offspring a survival advantage in unpredictable or novel environment (Shama, 2015). We have previously demonstrated that females exposed to chronically stressful conditions throughout a protracted breeding season adapt their reproductive strategy by producing larger offspring towards the end of the breeding season (Magierecka et al., 2022). It is therefore possible that females may adopt a bet-hedging strategy both within and between reproductive bouts.

Thus, 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 until relatively recently it has been difficult to measure the effects of stress exposure (in mothers) or maternal stress (in offspring) due to methodological limitations such as the requirement for stressful (or destructive) blood sampling and low-resolution methods of hormone quantification (Scott and Ellis, 2007; Sebire et al., 2007). In addition, the most widely used methods of inducing or mimicking maternal stress effects involve exposure of mothers to simulated predation (Feng et al., 2015), acute stressors (Sopinka et al., 2014, 2017) or exogenous GCs (Eriksen et al., 2015; Espmark et al., 2008), as well as exposure of eggs to exogenous GCs directly (Best et al., 2017; Sloman, 2010). However, exposure to acute stressors is unlikely to produce the same effects as chronic stress exposure. Whilst short-term exposure to stressors tends to confer a fitness advantage by inducing adaptations to cope with the stressful stimuli, chronic stress often carries a high physiological cost in terms of immune suppression and a shift in the reproduction/self-maintenance trade-off (Lattin and Kelly, 2020; Vitousek et al., 2019; Zimmer et al., 2019). The current pace of environmental change is likely to result in persistent low-intensity stress for many wild populations, as they are faced with an environment that is increasingly suboptimal for them (Alfonso et al., 2021; Cunningham et al., 2021; Lowry et al., 2013); it is thus important to consider the effects of chronic maternal stress for a realistic insight into the consequences of these changes. 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 over time, in addition to the effects of the implantation process on the stress axis and offspring phenotype (Crossin et al., 2016; Dufty et al., 2002). Lastly, exposure of ova to exogenous GCs as a proxy of heightened maternal stress levels only provides information on the effect of these hormones, without consideration of the other factors, such as maternal mRNAs (Colson et al., 2015; Sopinka et al., 2017).

In this study, we used three-spined sticklebacks (*Gasterosteus aculeatus*) from an annual population in which reproduction is confined to a single protracted breeding season (Lee et al., 2012) to explore the effects of chronic exposure to a stressful and unpredictable maternal environment on offspring behaviour and stress physiology. Our primary aim was to determine whether exposure of mothers to chronic unpredictable and stressful environmental conditions affected the behavioural phenotype of their offspring. Furthermore, we investigated whether behavioural and hormonal consequences of chronically stressful maternal environmental conditions differed between the offspring originating from successive clutches produced across the breeding season. To the best of our knowledge, this is the first study to explore the maternal effects of chronic stress in offspring from multiple clutches produced throughout the entire reproductive lifespan of a female.

2. Methods

2.1. Maternal stress protocol, in vitro breeding and fry husbandry

All animal work presented in this paper complied with the U.K. Animals (Scientific Procedures) Act 1986 (Project licence no. P89482164) and was approved by the University of Glasgow Animal

Welfare and Ethical Review Board.

The three-spined stickleback offspring used in this study were produced through in vitro fertilisation of eggs from adult females that were either exposed to an Unpredictable Chronic Stress Protocol (UCSP-exposed; Magierecka et al., 2021) or were non-exposed (Control females). These parental females were caught in the wild and transferred to an aquarium facility, where they were randomly allocated to one of the two treatments (UCSP-exposed $n = 72$; Control n = 72) and placed in groups of three (clearly differing in size to aid visual identification) in 10 L plastic tanks. Fish from the UCSP-exposed group were subjected to stressors throughout the breeding season and the protocol stopped once all females had ceased to reproduce (day 67). The daily UCSP schedule was constructed by randomly selecting three stressors from the list provided in Fig. 1. We also randomised the timing of presentation of each daily stressor, so that they were presented between 8 AM and 11 AM, between 11 AM and 2 PM or between 2 PM and 5:30 PM (except for stressor 1 presented at night). Randomisation of both the type of the stressor and the timing of its presentation, along with the prolonged nature of the stress protocol, allowed creation of a consistently unpredictable (and thus chronically stressful) environment. The tanks containing UCSP-exposed fish were physically separated from the tanks containing Control fish and fully shielded with opaque black plastic, so that the Control fish were not affected by any stressors. The UCSP did not result in elevated GC levels but caused a significant and persistent change in the behaviour and activity of the adult females, with no evidence of habituation over the course of the breeding season (Magierecka et al., 2021).

Throughout the experimental period we performed daily visual inspections of females from both treatments, and any females exhibiting signs of readiness to spawn (expanded abdomen and dilated anal papilla) were immediately lightly anaesthetised in 25 mg/L benzocaine solution so that their eggs could be stripped and fertilised in vitro following an established protocol (Barber and Arnott, 2000), using sperm from a randomly selected non-stressed stock male. After recovery from anaesthesia, females were released into their treatment tank, where they remained until they had produced a further two clutches (fertilised in the same way) or had ceased to reproduce. Each male fertilised only a single clutch, so that separate clutches from the same female were half-siblings. The fertilised eggs were kept in separate tanks until hatching.

Newly hatched fry were kept in their original family groups in 10 L plastic tanks (17x19x32 cm) fitted with an air stone for aeration as well as a plastic plant and a piece of black PVC pipe for shelter. Due to space constraints the size of each full-sibling family was reduced on day 60 to 15 fry where required. 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) and subjected to normal husbandry practices, with no additional stressors.

Behavioural and hormonal assays were then conducted on these offspring from the two treatment groups: note that the offspring themselves were not exposed to stressor treatments and so the terms 'UCSP group' and 'Control group' refers to the treatment experienced by their mothers. Assays were restricted to the offspring of females that produced at least two clutches during the breeding season, to allow for a comparison between the clutches within each treatment and to account for potential confounding factors that might have caused females to produce only one clutch during the breeding season. In addition, we used families with at least three surviving juveniles at the time of each assay to account for potential social aspects that could alter behaviour and stress physiology.

2.2. Behavioural tracking

To measure the effect of maternal stress exposure on offspring behaviour, we performed behavioural observations of 215 individual fish from 73 families ($n = 35$ families derived from UCSP-exposed mothers; $n = 38$ families from Control mothers) using Lolitrack Quattro video tracking software (version 1.00, Loligo Systems, Viborg, Denmark). The mean age of fish from the UCSP-exposed and Control groups was 275 days post hatch (dph, range: 253–306 dph) and 277 dph (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. Each arena was filled with aquarium water at 12 °C to a depth of 20 mm, 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 and illuminated from above by two 32 cm LED lighting strips producing 50 lm 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 four trials, carried out in immediate succession: (1) Open field trial (OF) – fish were placed individually in an empty arena and their movement recorded over the following 10 mins; (2) Novel object trial (NO) – an object with which the fish was not familiar (clear plastic ring 3 cm in diameter) was lowered into the arena and the movement of the fish recorded over the following 10 min; (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

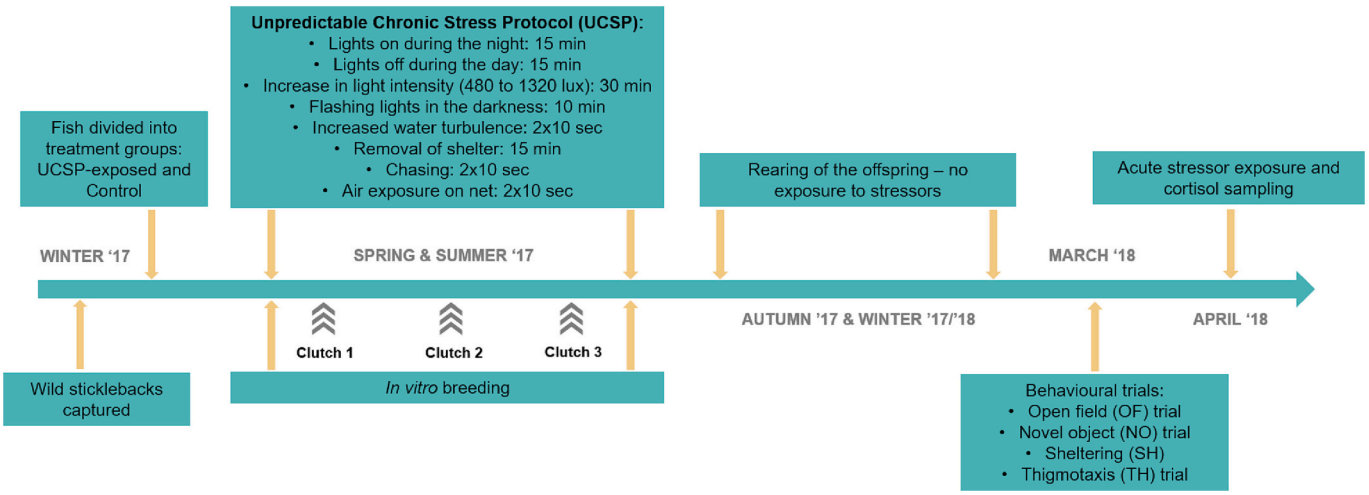

Fig. 1. Summary of the experimental timeline, including the outline of the stressors used in the Unpredictable Chronic Stress Protocol (UCSP).

only movement of the fish outside of the shelter was recorded over the following 10 min; (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 over the following 10 min.

The above set of observations was performed on three randomly selected fish per family, with each of the three fish tested on a separate day. The minimum time period to complete the tests for each family was 4 days and the maximum was 14 days. Each tested fish was placed in a temporary tank after testing to avoid observing the same fish more than once. The water temperature was recorded after each set of trials (range recorded: 12.0–13.1 °C), and water was replaced in the arenas before the next fish was tested so as to remove olfactory cues.

Live tracking was performed at a rate of 15 video frames per second. 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. A custom-designed R function was used to convert these coordinates into the following information: distance travelled (in m) and the proportion of time spent moving in both the OF and the NO trials, proportion of time spent at the perimeter of the arena in the TH trial and the probability of being in the shelter in each of the recorded frames during the SH trial.

2.3. Cortisol sampling and measurement

To measure the effect of maternal exposure to stressors on offspring stress-induced cortisol levels, we exposed a total of 204 offspring from 72 families ($n = 35$ families derived from UCSP-exposed mothers; $n = 37$ families from Control mothers; the families used in the cortisol trial were the same as the ones used in the behavioural trial) to an acute stressor and collected water borne cortisol samples. The mean age at the time of testing of offspring originating from the UCSP-exposed and Control group was 308 dph (range: 284–326 dph) and 304 dph (range: 276–330 dph) respectively. The non-invasive water sampling procedure was based on that described by Sebire et al. (2009, 2007) and Magierecka et al. (2021). Each fish was captured using a hand net and exposed to air while in the net for 2 × 2 min, with a 2 min rest in water between the exposures. This has been established to induce acute stress response in fish (Magierecka et al., 2021; Ramsay et al., 2009). 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 min, with peak cortisol release expected between 30 and 60 min (Sebire et al., 2007).

The water samples were transferred to 50 mL polypropylene tubes and stored at −20 °C until further analysis. Between uses, the beakers were rinsed with distilled water and 100 % methanol, and thoroughly dried. Prior to cortisol extraction, the samples were defrosted and brought up to room temperature; the water samples from all fish from the same family were pooled and processed as one sample to obtain an average cortisol concentration per family; the pooling of samples was deemed appropriate since sticklebacks from the same family group have been shown to co-regulate their cortisol release (Fürtbauer and Heistermann, 2016). Individual solid-phase extraction cartridges (Sep-Pak C18 Plus Light, Waters Corporation, Milford, MA) were primed with 1 mL of 100 % methanol and 1 mL of distilled water and placed on a 20-port vacuum manifold (VacMaster 20, Biotage, Uppsala, Sweden). The water samples were processed through the cartridges at a rate of 2 mL/min, and a sample spiked with 1.25 μL/mL of cortisol was processed in the same way to establish the extraction efficiency. The cartridges were then washed with distilled water and 20 % methanol and extracted with 1 mL of 100 % methanol into 12 × 75 mm borosilicate tubes. Extracted samples were evaporated under nitrogen gas in a sample concentrator with a heating block at 45 °C (Sample Concentrator with DB-3 Dri-Block, Techne, Stone, U.K.), diluted 1:3 in an assay buffer (supplied with the kit) and assayed in duplicate using a colorimetric competitive ELISA assay (ADI-900-071, Enzo Life Sciences, Exeter, U.K.). We assayed the same reference sample on each plate to determine inter-assay coefficient of variation (CV). Samples with the intra-assay coefficient of variation between the duplicates of >19 % ($n = 6$ out of 72 assayed samples) 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 %, which is expected due to a cross-reactivity of the cortisol ELISA with other steroid hormones.

2.4. Data analysis

All statistical analyses were performed in R statistical software (version 3.4.3, R Development Core Team).

The four measurements obtained from the open field (OF) and novel object (NO) trials (distance travelled and the proportion of time spent active in each trial) were highly correlated (Table S1 of the electronic supplement) and were hence combined in a Principal Components Analysis (PCA). The measurements were first z-score standardised using the "sapply" function in R Base Package to account for differences in standard deviation among the four measurements. The first principal component (PC1) extracted from the PCA 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: activity OF = 0.499, distance OF = 0.501, activity NO = 0.500, distance NO = 0.500). 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. PC1 was thus used as an index of activity in further analyses, with higher PC1 scores indicating higher overall activity levels, with fish spending more time in motion and covering a greater total distance during the trial.

A linear mixed model (LMM) was fitted using "lme4" package (Kuznetsova et al., 2017) to compare the activity (PC1) in offspring of UCSP-exposed ($n = 103$) and Control ($n = 112$) females, and between the offspring from the successive clutches produced by these females during the breeding season (Clutch 1 $n = 84$ offspring, Clutch 2 $n = 69$, Clutch 3 $n = 62$). The following fixed factors were included in the initial model: maternal treatment (categorical), clutch number (categorical), size of the family group (covariate; group size ranged 4–16 individuals in the UCSP-exposed group and 3–15 individuals in the Control group), age (covariate) and mass (covariate), as well as the maternal treatment*clutch number interaction. In this and all subsequent models, Maternal ID was included as a random intercept whenever there were multiple measurements (clutches) from the same female.

The scores for thigmotaxis (TH) and sheltering (SH) were not correlated with the majority of our measures of activity (see Table S1) and were thus not included in the PCA. A zero-inflated beta-binomial model with the same fixed and random factors as in the activity model was fitted to the TH data using "glmmTMB" package (Brooks et al., 2017). Zero-inflated beta models cannot handle outcomes of 1, and TH scores included a number of 1 s as an outcome; thus, in order to fit this model, the proportion of time spent around the perimeter of the arena was inverted (i.e. 1-outcome was calculated, so that outcome = 1 became outcome = 0). Therefore, the response variable in this model was the time spent in the centre of the arena, with fish spending all of the trial at the perimeter of the arena having a score of 0 and fish spending the majority of the trial in the centre of the arena having a score close to 1. "glmmTMB" with binomial distribution was used to analyse the proportion of time spent in the shelter, with the same fixed and random factors as the previous models and an additional random intercept of

observation-level random effect (OLRE) to account for overdispersion in the sheltering data.

The Intra-class Correlation Coefficient (ICC) of the behavioural scores, both within the clutches (i.e. for full siblings) and among all of the offspring produced by each female regardless of the clutch (i.e. for half-siblings), was analysed using the "rptR" package in R (Stoffel et al., 2017). ICC scores were translated into an intra-family variability of behaviour, where a high ICC value was associated with low variability among siblings or half-siblings, and vice versa.

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. "glmmTMB" model with gamma distribution was used to analyse the difference in mean peak cortisol levels between the families produced by UCSP-exposed ($n = 34$) 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), clutch number (categorical) and size of the family group (covariate), as well as the interaction term maternal treatment *clutch number; maternal ID was included as a random intercept.

3. Results

3.1. Behavioural scores

There was no significant difference in activity levels and thigmotaxis between offspring of UCSP-exposed and Control females ($F_{1,24.3} = 0.277$, $p = 0.603$ and X^2 (1, $n = 215$) $= 0.511$, $p = 0.475$, respectively), nor between offspring from the successive clutches produced by these females over a breeding season ($F_{2,152.7} = 0.962$, $p = 0.384$ and X^2 (2, n $= 215$) $= 0.123$, $p = 0.940$; see Table 1 for full regression models). The interaction between treatment and clutch number was also non-significant for both activity and thigmotaxis ($F_{2,196.0} = 0.254$, $p = 0.776$ and X^2 (2, n $= 215$) $= 0.490$, $p = 0.783$, respectively), as was the size of the family group ($F_{1,196.5} = 0.588$, $p = 0.444$ and X^2 (1, n $= 215$) $= 0.063$, $p = 0.802$), age ($F_{1,49.8} = 2.013$, $p = 0.162$ and X^2 (1, n $= 215$) $= 2.014$, $p = 0.156$) and mass ($F_{1,202.6} = 0.855$, $p = 0.356$ and X^2 (1, n $= 215$) $= 0.098$, $p = 0.754$). However, the maternal stress treatment had a clear effect in increasing the within-clutch variability in behaviour of the offspring, since activity score (PC1) was repeatable for both siblings and half-siblings in the Control but not in the UCSP-exposed group (Fig. 2, Table S2). Thigmotaxis showed very low ICC values in both treatment groups (Table S2).

The proportion of time in the shelter was independent of maternal treatment (X^2 (1, n $= 215$) $= 0.197$, $p = 0.657$), clutch (X^2 (2, n $= 215$) $= 0.075$, $p = 0.963$), the size of the family group (X^2 (1, n $= 215$) $= 3.163$, $p = 0.075$), age (X^2 (1, n $= 215$) $= 1.224$, $p = 0.269$) and body mass (X^2 (1, n $= 215$) $= 1.576$, $p = 0.209$; see Table 1 and Fig. S1). As with activity, sheltering behaviour was found to be repeatable in the Control but not in the UCSP-exposed group (Fig. 1, Table S2).

3.2. Cortisol response to an acute stressor

Offspring of those females that had been subjected to a period of chronic stress did not show an increase in stress-induced cortisol level as compared to offspring of Control mothers (X^2 (1, $n = 71$) $= 1.097$, $p = 0.295$), and the cortisol level did not differ between the offspring from the successive clutches produced by the females over a breeding season (X^2 (2, n $= 71$) $= 3.615$, $p = 0.164$; Table 2, Fig. 3).

4. Discussion

The results of our study show 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, we found a clear effect of maternal stress exposure on among-individual differences in behaviour, both within and between clutches, with an unpredictable and stressful maternal environment reducing the consistency of behaviour between both siblings and half-siblings. In this study, we also attempted to bridge the existing knowledge gap by investigating how chronic maternal stress affects offspring from different reproductive attempts of a given female. However, we found no inter-clutch differences in behaviour (either in interaction with maternal stress treatment or independently) or peak cortisol levels following exposure to an acute stressor.

These results did not provide evidence of directional 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 when only mean values for offspring behavioural phenotype were considered. These results are at odds with the current evidence from a range of taxa, where maternal stress effects (or pre-natal exposure to exogenous stress hormones 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 stress hormones (Best et al., 2017; Colson et al., 2015; Sopinka et al., 2015). Conversely, exposure of mothers to cortisol/physical stressors has been reported to result in reduced offspring activity under various scenarios (Eriksen et al., 2011; Espmark et al., 2008;

Table 1
Summary of the models used to analyse the differences in activity (PC1; random intercept variance: 0.281, marginal R^2: 0.034, conditional R^2: 0.112), thigmotaxis (TH; random intercept variance (0.000, R^2: 0.301, adjusted R^2: 0.270) and proportion of time spent in shelter (random intercept variance: 1.84 for maternal ID, 18.87 for OLRE; marginal R^2: 0.050, conditional R^2: 0.122) in juveniles from three successive clutches produced by Control and UCSP-exposed female three-spined sticklebacks during the breeding season. Values give estimates ± SE. 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 all models as a random intercept, with the sheltering model containing an additional random intercept of observation-level random effect (OLRE). Control group $n = 112$ (Clutch 1 $n = 44$, Clutch 2 $n = 36$, Clutch 3 $n = 32$) and UCSP-exposed group $n = 103$ (Clutch 1 $n = 40$, Clutch 2 $= 33$, Clutch 3 $= 30$).

Model terms	Effect on activity (PC1)	Effect on TH score	Effect on prop. of time in shelter
Maternal treatment	−0.035 ± 0.444, $t_{1,73.4} = -0.080$, $p = 0.937$	0.148 ± 0.170, z $= 0.871$, $p = 0.384$	−0.155 ± 1.097, z $= -0.141$, $p = 0.888$
Clutch 2	0.196 ± 0.498, $t_{2,192.6} = 0.394$, $p = 0.694$	0.036 ± 0.203, z $= 0.176$, $p = 0.860$	−0.012 ± 1.225, z $= -0.010$, $p = 0.992$
Clutch 3	−0.437 ± 0.536, $t_{2,157.4} = -0.816$, $p = 0.416$	0.051 ± 0.222, z $= -0.227$, $p = 0.820$	0.009 ± 1.327, z $= -0.007$, $p = 0.995$
Mat. treatment *Clutch 2	−0.400 ± 0.601, $t_{2,195.2} = -0.666$, $p = 0.506$	−0.047 ± 0.244, z $= -0.192$, $p = 0.848$	−0.182 ± 1.473, z $= -0.123$, $p = 0.902$
Mat. treatment *Clutch 3	−0.032 ± 0.611, $t_{2,194.1} = -0.052$, $p = 0.959$	−0.177 ± 0.257, z $= -0.687$, $p = 0.492$	−0.579 ± 1.501, z $= -0.386$, $p = 0.699$
Family group size	0.030 ± 0.039, $t_{1,196.5} = 0.767$, $p = 0.444$	−0.004 ± 0.015, z $= -0.251$, $p = 0.802$	−0.172 ± 0.097, z $= -1.778$, $p = 0.075$
Age	−0.020 ± 0.014, $t_{1,49.8} = -1.419$, $p = 0.162$	−0.007 ± 0.005, z $= -1.419$, $p = 0.156$	0.040 ± 0.036, z $= 1.106$, $p = 0.269$
Mass	0.000 ± 0.000, $t_{1,202.6} = 0.925$, $p = 0.356$	−0.000 ± 0.000, z $= -0.313$, $p = 0.754$	0.002 ± 0.001, z $= 1.255$, $p = 0.209$

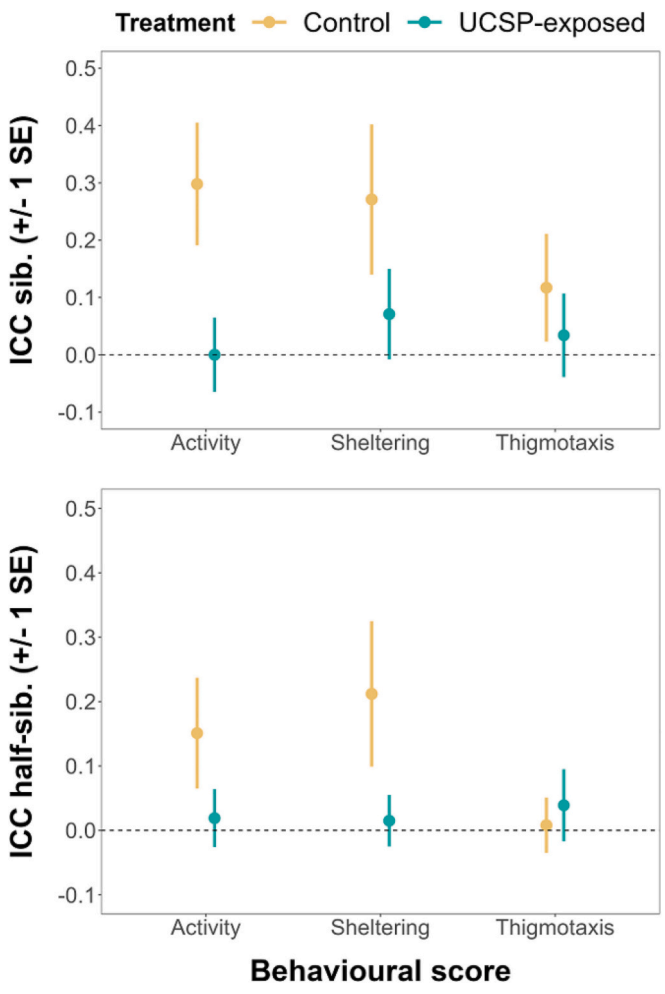

Fig. 2. Intra-class Correlation Coefficients (ICC) of the behavioural scores (activity (PC1), probability of sheltering, and thigmotaxis) in juveniles produced by three-spined stickleback females from Control (n = 112 from 38 clutches) and UCSP-exposed (n = 103 from 35 clutches) treatment groups. The top panel represents the ICC within the clutches (full-siblings); the bottom panel represents the ICC among all of the offspring produced by each female (half-siblings). Dashed line: ICC = 0 (i.e. between-individual repeatability no different from random).

Table 2
Summary of the model used to analyse the variation in peak waterborne cortisol level (in ng/mL) in juveniles originating from three successive clutches produced by Control and UCSP-exposed female three-spined sticklebacks during the breeding season. Values give estimates ± SE. The reference maternal treatment was the Control group, and the reference clutch was the first clutch in the season. Random intercept variance: <0.0001; $R^2 = 0.088$. Control group $n = 37$ (Clutch 1 $n = 14$, Clutch 2 $n = 12$, Clutch 3 n = 11) and UCSP-exposed group $n = 34$ (Clutch 1 $n = 13$, Clutch 2 = 11, Clutch 3 = 10).

Model terms	Effect on offspring peak cortisol
Maternal treatment	0.159 ± 0.249, z = 0.639, $p = 0.523$
Clutch 2	0.221, ± 0.252, z = 0.874, $p = 0.382$
Clutch 3	0.515 ± 0.256, z = 2.011, $p = 0.044$
Mat. treatment *Clutch 2	−0.326 ± 0.365, z = −0.893, $p = 0.372$
Mat. treatment *Clutch 3	−0.707 ± 0.372, z = −1.901, $p = 0.057$
Family group size	−0.024 ± 0.022, z = −1.066, $p = 0.287$

O'Brien et al., 2017; but see Colson et al., 2019). Other aspects of offspring behaviour that can be affected by maternal stress include the propensity to remain close to the walls whilst in an open space (thigmotaxis), often used as an indicator of anxiety levels (Simon et al., 1994); this tends to be negatively correlated with maternal stress/exogenous GC exposure (Best et al., 2017; Redfern et al., 2017; but see Kapoor and Matthews, 2005). 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 (Ensminger et al., 2018; Uller and Olsson, 2006).

A plausible explanation of the lack of chronic maternal stress effect observed in our study may be related to the mechanism through which mothers adjust offspring behaviour in response to stress if this involves altered GC levels. An earlier study on the effects of an unpredictable and stressful environment in sticklebacks found evidence of behavioural but not hormonal responses of females to chronic stress (Magierecka et al., 2021). It is thus possible that the stickleback offspring did not experience elevated cortisol levels *in ovo*. 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 a consistently 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 et al., 2018). Moreover, behavioural trials incorporating the context of offspring behavioural response to stress may provide better insight into the link between chronic maternal stress and offspring behaviour compared to testing the offspring in a benign environment.

Even though the offspring behavioural phenotype did not seem to differ directly between the offspring of UCSP-exposed and Control females, exposure of mothers to an unpredictable and stressful environment increased the between-individual variability of two of the three measured behavioural traits in their offspring, an effect that was consistent both within and between the breeding attempts (clutches) of the same female. Whilst repeatability of individual behaviour is well documented, including in the context of maternal effects (Bell et al., 2009; Reddon, 2012), rather few studies have quantified the between-individual repeatability of behavioural phenotypes in individuals from the same family, particularly in response to maternal stress. However, our results are congruent with studies showing that environmental perturbations result in increased intra-family phenotypic and behavioural variation (Oldham et al., 2019; Shama, 2015). The increased between-individual variation in offspring behaviour, as observed in our study, may be an example of an adaptive maternal effect, whereby mothers hedge their bets by producing offspring differing in behavioural phenotype, and thus increase the chance that some of these offspring will be able to better cope with the prevailing conditions, resulting in an overall increase of offspring fitness in an unpredictable environment (Crean and Marshall, 2009). Our finding thus provides the ground for further studies on maternal stress and the consistency of offspring behavioural traits between related individuals, which are currently scarce. Since the effects we observed are unlikely to be driven by GCs, these studies should consider non-GC-based mechanisms behind maternal effects, such as differential allocation of androgens or maternal mRNAs deposited in the developing oocyte.

In addition, the behaviour of offspring in our study was largely independent of the clutch from which they originated, with little difference between early- and late-produced clutches. This is despite the finding of Magierecka et al. (2022) that the reproductive investment of female sticklebacks varied over the sequence of clutches and was influenced by exposure of females to chronically stressful environmental conditions, providing a reason to predict that an interaction between clutch order and maternal environment can affect offspring behaviour. There is however a lack of comparable studies looking at inter-clutch differences in offspring behaviour.

The hormonal response to an acute stressor of juvenile sticklebacks

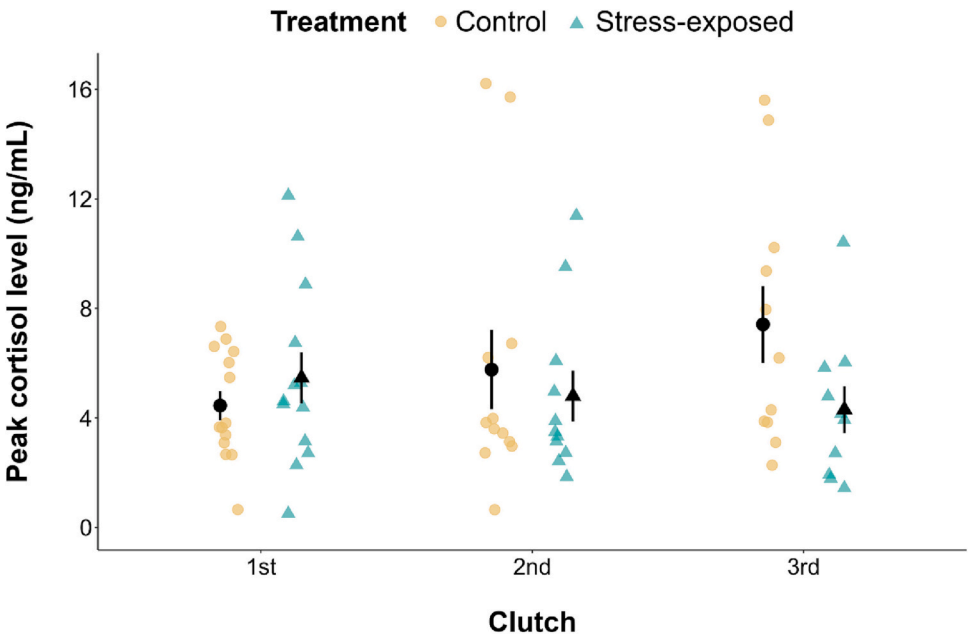

Fig. 3. Differences in mean peak waterborne cortisol levels following the exposure to an acute stressor (in ng/mL) in juveniles originating from three successive clutches produced across the breeding season by three-spined stickleback females from Control (n = 37; Clutch 1 n = 14, Clutch 2 n = 12, Clutch 3 n = 11) and UCSP-exposed (n = 34; Clutch 1 n = 13, Clutch 2 = 11, Clutch 3 = 10) treatment groups. Data shown as individual data points (offset for clarity) together with mean values plus error bars representing Standard Error of the Mean (SE).

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 to either chronic or acute stressors had an effect on offspring stress physiology. For example, chronic maternal social stress resulted in increased baseline cortisol levels of offspring (Bian et al., 2015), whilst reduced baseline cortisol was observed with both chronic and acute maternal stress (Kleist et al., 2018; Tilgar et al., 2016). 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 (Jeffrey and Gilmour, 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 and the degree to which it is regulated through negative feedback may be altered (Mommer and Bell, 2013; Tilgar et al., 2016; Zimmer et al., 2019). It is important to stress that in this study we had no information on baseline cortisol level (and thus on the magnitude of cortisol increase following the exposure to an acute stressor), which may explain why we did not observe any effect of maternal exposure to stressors on offspring cortisol level, as evidenced in other studies. In future studies it would be beneficial to measure offspring baseline cortisol level, as well as their hormonal responses at different time points after the acute stress exposure, and to address the dynamic regulation of GCs through negative feedback. It is unclear whether the lack of effect of maternal stress exposure on 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 our results if an anticipated unpredictable offspring environment favoured greater flexibility of physiological stress responses.

5. Conclusions

In this paper we report no direct effect of chronic maternal exposure to stressors on offspring behaviour, but a clear effect on within-family diversification of behavioural traits. This indicates that maternal effects on offspring behaviour are complex in nature, such that it may be critical to consider the same behaviours from various angles when examining the effects of maternal stress. Interestingly, but quite unexpectedly in light of the current state of knowledge, offspring of females exposed to an unpredictable environment had neither increased nor reduced stress-induced cortisol levels in response to an acute stressor. Our analysis of the differences in behaviour and stress-induced cortisol levels in offspring from successive clutches produced by annual fish across their reproductive lifespan shows lack of seasonal variation in offspring traits, despite previous evidence of inter-clutch differences in maternal investment.

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2023.105396.

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