Heatwave-mediated maternal effects: heat stress in late but not early pregnancy has carry over effects on offspring phenotype

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Abstract

Heatwaves are extreme climatic events driven by climate change that profoundly affect animal survival and reproduction. Although evidence for the direct effects of heatwaves on reproduction is increasing, much less is known about whether heatwaves during pregnancy generate maternal effects on offspring, and whether these effects depend on the timing of exposure. Pregnancy is a metabolically demanding life-history phase, with energetic demands changing markedly across gestation. We experimentally exposed pregnant female guppies (*Poecilia reticulata*), a small viviparous tropical fish, to a five-day simulated heatwave during either early or late pregnancy. We quantified maternal reproductive success and offspring performance, behaviour and sexually selected traits. Brood size did not differ across groups, but maternal heat exposure in late pregnancy was associated with body size-dependent effects on within-brood mortality; larger females exposed to heat late in pregnancy were more likely to experience offspring mortality. Despite no detectable effects on offspring growth, late gestational heatwaves altered offspring behaviour and adult offspring phenotype. Offspring of late-exposed mothers exhibited increased exploratory behaviour and swimming velocity measured in an open field assay, whereas overall swimming performance was unaffected, suggesting that these behavioural differences were not explained by altered swimming capacity. Maternal heat exposure also altered the degree of within-treatment variability in some phenotypic traits, suggesting that heatwaves may influence not only mean offspring phenotype but also degree of phenotypic variation. Moreover, adult male offspring (F1) from late-exposed mothers displayed a greater extent of orange body coloration, a key sexually selected trait in guppies, whereas sperm traits were unaffected in all descendant males. Overall, our findings demonstrate that heatwaves during gestation, and especially during its late stages, can alter females' reproductive success and shape the development of offspring behavioural and sexually-selected traits via maternal effects. The developmental timing of maternal heat exposure is a decisive factor determining the magnitude and nature of maternal effects, with consequences that persist until sexual maturity. By influencing exploration and sexual traits, heatwave-mediated maternal effects may alter offspring fitness and population responses to increasingly frequent climatic extremes, and may produce cross-generational effects extending beyond directly exposed individuals.

Keyword: maternal effects, transgenerational effects, non-lethal effects, extreme climatic events, heat stress, timing of reproduction, open field, exploration, critical swimming speed, fecundity, carry over effects

Introduction

Heatwaves are short but intense periods of extreme temperatures, typically lasting only few days (Perkins-Kirkpatrick & Lewis, 2020). These extreme events linked to climate change are increasing globally both in frequency and intensity (Luo et al., 2024) and have been associated with reduced survival in animals and humans as well as with numerous mass mortality events (Jones et al., 2018; Piatt et al., 2020; Xu et al., 2016). Beyond their impact on survival, heatwaves can severely disrupt reproductive processes, sexually-selected traits and mating behaviour (Morosinotto et al., 2025). Fertility is a thermal sensitive trait that is known to be strongly affected by temperature (Walsh et al., 2019), even when several degrees milder than lethal temperatures (Bretman et al., 2024; van Heerwaarden & Sgrò, 2021). Exposure to heatwaves reduces gametes survival and quality (Breedveld et al., 2023; Sales et al., 2018; Sales et al., 2021; Vasudeva et al., 2021). Beyond direct effects on exposed individuals, thermal stress experienced during reproduction may also generate parental effects that shape offspring phenotype and performance (Morosinotto et al., 2025). Indeed, stress-mediated parental effects can induce persistent effects on offspring growth, physiology and behaviour (Marshall & Uller, 2007; Sheriff & Love, 2013).

The timing of exposure to heatwaves is a critical determinant of their biological impact as the magnitude of the effects can vary substantially depending on whether thermal stress occurs during gametogenesis, mating, embryonic development or offspring growth (Mejía & Wetzel, 2023; Morosinotto et al., 2025; Pilakouta et al., 2023). For example, in burying beetle (*Nicrophorus vespilloides*), heatwaves occurring during mating markedly reduced the likelihood of remating and affected offspring size and condition compared to exposure before mating or at hatching (Pilakouta et al., 2023). We can expect that heatwaves occurring just before or during mating will affect gametogenesis, sexual behaviour, and/or fertilization success (Breedveld et al., 2023; Morosinotto et al., 2025; Pilakouta et al., 2023). Heatwaves occurring during embryonic development could on the other hand affect hatching success and several aspects of offspring phenotype (Morosinotto et al. 2025), by altering for example the development of cognitive, endocrine, reproductive and locomotor systems. However, whether the consequences of heatwaves on embryonic development depend on the timing at which reproducing individuals are exposed to heatwaves remains largely unknown.

A particularly vulnerable reproductive stage is pregnancy, because females must simultaneously sustain their own metabolism and the increasing energetic demands of developing embryos. Live-bearing females experience elevated metabolic demands and increased locomotory costs compared to oviparous species (Fleuren et al., 2019; Ghalambor et al., 2004; Hussain et al., 2024), with these costs intensifying as pregnancy progresses. Indeed, toward the end of gestation, ectothermic

viviparous females exhibit up to threefold higher oxygen consumption (Foucart et al., 2014) and reduced thermal tolerance (Auer et al., 2021; Virens & Cree, 2019) compared to females in early pregnancy and to oviparous females. These changes reflect both the rapid increase in embryonic metabolic demand and the heightened maternal metabolic costs across gestation (Foucart et al., 2014). In addition to the high metabolic costs for pregnant females, embryos themselves may be particularly vulnerable to thermal stress because increased embryonic oxygen demand must be met within the physiological constraints imposed by the mother (Virens & Cree, 2019). Thus, due to the different degree of oxygen consumption and thermal tolerance of both females and embryos during gestation, we can expect that the impact of heat on both females stress response and embryo's development will depend on the timing of the heatwave during the pregnancy.

Moreover, embryos of viviparous species may experience prolonged exposure to maternal stress hormones, such as glucocorticoids, compared to embryos of oviparous species (MacLeod et al., 2021), potentially amplifying the consequences of maternal stress during development. In viviparous species, embryos develop within the maternal environment and are therefore exposed throughout gestation to maternal physiological conditions, including fluctuations in circulating hormones. In contrast, embryos of oviparous species are primarily exposed to maternal hormonal signals deposited into the eggs before oviposition. Thus, heat stress experienced during pregnancy may impose substantial costs on both mothers and developing embryos in live-bearing species. Understanding the effects of heatwave during pregnancy on offspring performance and fitness-related traits is thus crucial for predicting the vulnerability of viviparous animals to increasingly frequent and intense heatwaves.

Viviparous lecithotrophic fish, such as the guppy (*Poecilia reticulata*), are an ideal study system to investigate stress-mediated maternal effects during pregnancy. They represent a simplified model for pregnancy compared to mammals and especially compared to species that provide parental care. Since guppies provide no parental care, they offer the unique opportunity to isolate the effects and costs of pregnancy from those of postnatal parental investment, such as lactation, food provisioning, and predator defense, which may themselves be influenced by temperature. During pregnancy, embryonic nutrition is largely yolk-based (Campuzano-Caballero & Uribe, 2014; da Silva et al., 2023) but maternal physiological conditions still determine the thermal and oxygen environment experienced by developing guppy embryos. Indeed, respiration and excretion are accomplished by exchange throughout the follicular placenta (Uribe and Grier 2010**)** with some small amount of maternal transfer observed across gestation (DeMarais & Oldis, 2005). Embryonic oxygen availability and temperature exposure will be entirely mediated by the conditions experienced by the female during gestation and by maternal physiology. In Poeciliid fishes, heightened rate of oxygen

consumption and increase ventilation as pregnancy advances have also been recorded (Timmerman & Chapman, 2003), likely reflecting the increment in oxygen demands of developing embryos as they grow in size (Ghalambor et al., 2004). Consistent with elevated oxygen demands during late pregnancy, guppy females at advanced gestational stages exhibit a reduction in thermal limits of 0.5 °C compared to females in early pregnancy (Auer et al., 2021). Moreover, guppy embryos increase nearly fourfold in wet mass between conception and birth (Ghalambor et al., 2004) and females incur in substantial locomotory costs that progress through gestation (Ghalambor et al., 2004). Together, this evidence suggests that vulnerability to heat stress changes across pregnancy. Consequently, heatwaves occurring at different stages of gestation may generate distinct effects, because maternal physiological constraints and embryonic demands change over time.

In guppies, a recent study showed that females exposed to a simulated 5-day heatwave had lower survival and strongly impaired fertility (Breedveld et al., 2023). Also, in this species, stress-mediated maternal effects are well documented. Offspring of mothers exposed to mild stressors exhibit reduced associative learning ability and greater behavioural variability (Eaton et al. 2015). Predator-exposed mothers produced smaller offspring (Monteforte et al., 2020; Stein and Hoke 2022**)** with altered explorative behaviour (Cattelan et al., 2020; Stein and Hoke 2022) as well as sex-specific activity patterns (Leri and Stein 2024), suggesting that maternally perceived stress can be transferred to embryos. In live-bearing species, stress-mediated maternal effects refer to effects and responses in the offspring resulting from maternal exposure to stress during gestation. In viviparous ectotherms such as guppies, embryos are exposed to the same thermal environment as their mothers. Thus, when considering heatwave as stressors in these species, maternal effects comprehend both the effects mediated by maternal physiology and the possible effects of direct thermal exposure of embryos within the maternal reproductive tract.

Here we investigate the effects of heatwaves occurring during either early or late pregnancy on female fertility and on offspring phenotype, from birth till sexual maturation. Timing of heatwaves can be a strong driver of heatwave-mediated maternal effects: early gestation may represent a critical window for developmental embryonic programming, whereas late gestation may represent a period of physiological constraint due to increased embryonic growth and oxygen demand. By observing offspring growth, behaviour and sexual traits development we investigate the possible carry over effects of maternal stress to future generations. We expect heatwave exposure during pregnancy to impair embryo development and survival but also to induce persistent effects in adulthood, with these effects possibly varying depending on the stage of embryonic development at which thermal stress occurs.

Material and methods

Overview of the experiment

To investigate the effects of heatwaves at different stages of pregnancy on females' fertility and offspring phenotype, we randomly assigned females to three different treatment groups. After mating, females were exposed to a heatwave at either early phase of pregnancy (early group o HWE) or late phase of pregnancy (late group o HWL) or were maintained in control temperature conditions throughout pregnancy (control group o CTRL). Females in heatwave treatments were maintained at 30 °C during temperature manipulation while females in control treatment were constantly maintained at 26 °C. In a previous experiment, guppy females exposed to a heatwave had lower survival and were mostly not able to produce live broods preventing any assessment of effects on offspring (Breedveld et al. 2023). Thus, in this study, to be able to investigate reproductive success and maternal effects on offspring phenotype we lowered the heatwave temperature to which females were exposed (from 32 °C to 30 °C). Following the heatwave treatment, females were monitored until parturition to record gestation time, female reproductive success and offspring quality (Figure 1). Offspring development was monitored from birth to juvenile stages by combining morphological measurements (at 24 h and at 30 days of age), behavioural assays and swimming performance tests (at 30 days). Offspring quality at adulthood was assessed once the offspring reached sexual maturity, and traits measured included body size, in both sexes, and coloration (associated with male attractiveness) and sperm number and quality, in males.

Fish maintenance before the experiment

Fish originated from a self-sustaining population maintained in a large seminatural tank at the Botanical Garden of the University of Padova (approximately 4600 × 440 cm filled with 40 cm of water). Visibly pregnant females were collected and transferred to the laboratory facility, where fish were maintained under standard conditions (24–27 °C, 12-h light cycle, fed twice day *ad libitum* with dry food and live nauplii of *Artemia salina*, supplemented once a week with frozen chironomid larvae). Pregnant females, identified by the extension of the abdomen, were housed individually until parturition in 3.5 L tanks within a recirculating water system (Tecniplast) which maintains identical water quality (including temperature and pH) across all tanks. Females were isolated but in visual contact with conspecifics and tanks were checked daily to record parturition day.

Start of the experiment

Within 1-2 days from parturition, when females are receptive, each female was housed with three adult males in a 8 L tank within the Tecniplast system for a three-day mating period (days T-3 and

T0; Figure 1). Males were randomly collected and had no previous contact with the focal female. After the mating period, males were returned to stock tanks in the laboratory while females were photographed to measure body size (standard length, in mm) using ImageJ software (www.imagej.net; Schneider et al., 2012). Females were then assigned an experimental ID and isolated in plastic containers equipped with mesh windows, thereby allowing water flow in and out of the container as well as visual and olfactory contact with conspecifics (day T0 in Figure 1).

Heatwave treatment

Average gestation duration in this species for non-virgin females is 30 days (range 22-38 days, Gasparini and Evans 2018). Females assigned to the early heatwave treatment were thus exposed during the first week of gestation (mating between T-3 and T0 and heatwave just after mating, Figure 1). Females assigned to the late heatwave treatment were exposed toward the end of the pregnancy, approximately 15 to 19 days after fertilization (Figure 1). Control females underwent the same handling as the heatwave females, but water temperature was maintained at 26 °C throughout the experiment.

During the heatwave treatments, females were transferred to experimental tanks, acclimated at 28 °C for 2h to minimize thermal stress and then maintained at 30 °C for 5 days. Water temperature was controlled using aquarium heaters and monitored continuously with digital dataloggers (precision ±0.1 ºC; EnvLoggers T2.4 www.electricblue.eu). On the 5th heatwave day, females were acclimated at 28 °C for 2 h and then maintained at 26 °C for the rest of the study. Females in the control group were also transferred to experimental tanks that were identical to heatwave tanks except for the water temperature that was set at 26 °C. Females were housed throughout the experiment in plastic containers equipped with mesh windows that allowed visual and olfactory communication with other females undergoing the experiment simultaneously. Outside the 5-days temperature manipulation treatment, females remained in the same containers within stock tanks with other individuals swimming freely around the containers.

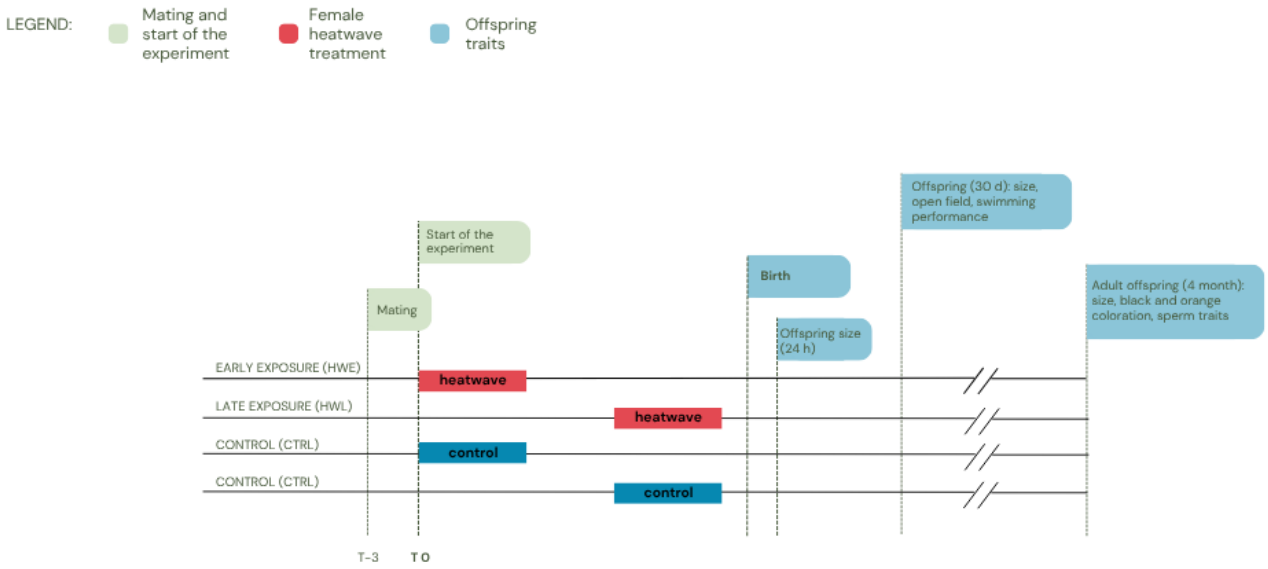

Figure 1: Diagram of the experimental design. Immediately after giving birth, females were placed for 3 days with males in order to mate (T-3 till T0), then were randomly assigned to one of three treatments: early exposure to heatwave (HWE), late exposure (HWL) or control (CTRL) group. Early exposure occurred between 3 and 7 days after mating while late exposure between 15 and 19 days after mating. Females in heatwave treatments (both early and late) were exposed to 30 °C for 5 days and then to 26 °C for the rest of the experiment, whereas females in the control group were maintained throughout at 26 °C. At birth, offspring were isolated, photographed at 24 h, and then kept until 30 days of age when they were photographed again, and then tested for swimming performance and behaviour in an open field. A subset of offspring were then kept until sexual maturation at 4 months and were photographed, male coloration was scored and sperm velocity and number estimated.

Females' reproductive success

At T16 (i.e. 19-20 days after mating) all females were isolated in 3.5 L tanks within the Tecniplast recirculating system and maintained at 26 °C in standard conditions (see above *Fish maintainance*) until parturition. Females were checked twice a day to determine the day of delivery and gestation duration, the total number of offspring were recorded. The proportion of live and dead offspring per brood was also monitored up to the 30 days of age of the offspring.

Juveniles' offspring phenotype: morphology, behaviour, and swimming performance

At birth, each brood was collected and transferred into a new 3.5 L tank where offspring remained until 1 month of age. Consequently, rearing density during this period varied among broods according to brood size and was accounted for in the statistical analyses. At 24 h after birth and again at 30 days of age (± 3 days), all offspring (from a subsample of broods, see Table S1) were photographed from

above using a stereomicroscope-mounted camera (LEICA S9D). A sheet of millimeter graph paper in each image was used for calibration, and standard length (mm) was measured using ImageJ (www.imagej.net; Schneider et al., 2012). At 30 days old (± 3 days), offspring also underwent behavioural and swimming performance assays. These assays were performed on a subsample of offspring (up to 10 per brood) for large broods due to methodological constraints; the tested offspring were randomly selected within the broods considered for these tests (Table S1).

Juveniles were tested in an open-field assay to quantify exploratory behaviour and antipredator responses. The open-field trial followed a modified protocol from Breedveld et al. (2025). Each juvenile was placed in a rectangular arena (26.5 cm x 19 cm x 6.5 cm) filled with 1 L of water, which was replaced after each trial. Juveniles were allowed to acclimate for 5 minutes in the arena and then their exploratory behaviour was recorded for 10 minutes under constant light conditions with a video camera (Panasonic HC-V180) placed above the arena. After this first 10 minutes, 50 µl of a chemical alarm cue was added to the arena and the behaviour of the juvenile was recorded for a further 10 minutes. Chemical alarm cue, simulating an attack to a conspecific, was prepared following protocols in (Breedveld et al., 2025; Cattelan et al., 2020)**.** Videos were analyzed using the software AnimalTA (version 2.3.4), to extract for each individual per each 10-minute phase: the proportion of arena explored, defined as the proportion between the area within 1cm of the individual's body center relative to the total area of the arena; the mean swimming velocity (cm s^{-1}; calculated only when swimming speed exceeding 1 cm s^{-1} to exclude passive drifting); and the overall activity (defined as the total distance moved during the behavioral test, cm; Chiara and Kim 2023).

After the behavioural trials, each juvenile was tested for swimming performance at 26 °C, following an established protocol (Breedveld et al., 2023). Briefly, each juvenile was placed in a cylindrical swim chamber (length 50 cm, 1.6 cm wide). Water flow was initially set at *7* cm s^{-1}. After one minute, the speed was increased by 3 cm s^{-1} every minute until reaching a maximum speed of 22 cm s^{-1} after 5 minutes. Water velocity then remained constant for the rest of the trial. The test ended when the fish was no longer able to swim against the current and was swept from the rear end of the chamber into a water tank below. Two juveniles kept swimming for over 8 minutes and thus the test was ended by the observer (2 juveniles on 346 total, one from control group and one from an early-exposed mother). The time spent swimming (s) was recorded taking into account the increment in water velocity and then critical swimming speed was calculated (see *Statistical methods*; Brett 1964; Breedveld et al., 2023).

Upon completion of the experimental trials, five offspring per brood (mean ± s.e. 4.57 ± 0.044 offspring per tank) were randomly selected and reared until sexual maturity (4 months of age). This

sample size was chosen for logistical reasons while also standardizing brood size across families, thereby minimizing variation in rearing density, which is known to influence growth rate in this species (Reznick et al. 2012 and this study). Offspring were reared in 3.5 L Tecniplast tanks within a recirculating system under standard conditions for the species, as described above.

Adult offspring phenotype: body size, coloration and sperm traits

Adult offspring phenotype was assessed after they reached sexual maturation at 4 months of age (± 15 days). Fish were photographed from above using a digital camera (Canon EOS 400D) and under standardized lighting conditions to measure standard length (mm) using Image J (www. imagej.net; Schneider et al., 2012). Each image included a millimetric scale for calibration and a Pantone colour chart for colour calibration. Male coloration was quantified from photographs by measuring the area of orange/yellow and black spots using ImageJ. Reflectance spectra (between 400 and 700 nm) were also estimated from the images using the software ColourWorker (https://www.colourworker.com/), using guppy-specific orange spectra as reference values **(**Gasparini et al. 2014). From each male two-three different orange spots were measured and the average spectrum was then calculated. Values of brightness and orange chroma were then measured. Brightness was obtained by calculating the area of the total amount of reflected light by the male ornaments along the whole spectrum. Orange chroma is the proportion of the sum of the reflection from 575 to 700 nm from yellow/orange to red on the total reflections on the whole spectrum.

Sperm traits were assessed in male offspring following standard protocols for this species (Breedveld et al., 2023; Gasparini et al., 2017). Briefly, males were sedated with MS-222 and placed under a stereoscope. Gentle pressure was applied to the abdomen to collect sperm in physiological solution (0.9 %). Sperm motility was assessed immediately after sperm collection using computer-assisted sperm analysis (CEROS Sperm Tracker version 12.3, Hamilton Thorne Research, Beverly MA) from which swimming velocity (VCL) was extracted. Sperm motility was estimated on a mean of 123.99 ± 4.023 (range 12-351) sperm cells. Sperm number (i.e. the total number of sperm in the ejaculate) was estimated using the same computer-assisted sperm analysis using a Leja chamber (20µm). To avoid that sperm number could be affected by recent mating, all male offspring were isolated 48h before sperm traits assessment using a transparent separator that allowed visual and olfactory contact with the females within the same 3.5 L tank.

Statistical methods

All the analyses were performed in R version 4.6.1 (R core Team 2026) and RStudio version 2026.01.1. Linear or generalized models were performed using packages *lme4* and *glmmTMB,* as

described for each variable. In all the models Anova function (Wald χ^2 test, package *car*) were used to assess the significance of fixed effects. Type III tests were used when models included significant interactions; otherwise Type II tests were applied. Statistical significance was set at $\acute{\alpha}$=0.05. Significant treatment effects were explored using pairwise contrast analyses estimated with the *emmeans* package, whereas simple slopes analyses were conducted using *emtrends* package if interactions between treatment and another fixed effect were found. Model simplification was performed by comparing models using Akaike's Information Criterion (AIC). Figures were produced using *ggplot2, sjPlot,* and *interactions.* Residuals diagnostics was performed with package *DHARMa*.

Sample size varied among analyses due to differences in reproductive success and occasional missing measurements resulting from methodological constraints. Overall, 116 females entered the experiment, of which 96 gave birth. Gestation duration was recorded for 92 females, while offspring number and mortality were available for 91 broods. Offspring phenotype, both as juveniles and adults, was analyzed for a subsample of broods. Sample sizes for each analysis are reported in Supplementary Table S1.

a) Females' reproductive success

In all the models related to females' reproductive success (i.e. the probability of delivering the first brood, gestation duration, number of offspring, offspring mortality and the number of alive and dead offspring), treatment and female body size (i.e. standard length), as well as their interaction, were included. The interaction between treatment and body size was removed from a final model if $P > 0.1$ and if the AIC of the model without interaction was smaller (i.e. improved model fit, result not shown) thus allowing interpretation of the main effects. Probability of delivering a brood (yes/no) and probability of offspring mortality (0 = no mortality,1 = at least one dead offspring) were analyzed as binary response variables using generalized linear models (glm) with binomial distribution and logit link function. An additional model testing the probability of offspring mortality included brood size as a covariate to account for any stochastic effects associated with a higher probability to have dead offspring in larger broods. As brood size was positively correlated to female body size ($r = 0.537$, $P = <.001$), which was already included in the model, and did not qualitatively change the results, only the simpler model is presented. The number of dead and live offspring at 30 days of age was analysed in a glmmTMB model fitted with betabinomial family. Brood size (i.e. the total number of offspring produced) was analyzed using a generalized linear model with a negative binomial distribution (glm.nb). Gestation duration was analysed using a linear model with gaussian distribution.

b) Juvenile offspring phenotype

Offspring body size at 24 h after birth, at 30 days of age, and after sexual maturation were analysed in three separate linear mixed models with the lmer function. Residuals followed a gaussian distribution in all three models. For the model of offspring size at 24 h and at 30 days, the interaction between maternal treatment and brood size, initially included in the model, was excluded because of non-significance ($P > 0.1$, see above). In these models, mother identity (hereafter female ID) was included as random factor to control for non-independence of data collected from siblings.

Offspring behaviours (exploration, swimming velocity and overall activity in the open field) were analyzed according to phase (pre- and post- exposure to alarm cue), maternal treatment (CTRL, HWE, HWL) and brood size at 30 days old. All the models included the interaction between phase and treatment, which was removed if $P > 0.1$. Female ID and offspring ID were included as random effects, to consider non-independence of siblings and repeated measures within individuals. Exploration behaviour, i.e. the proportion of arena explored during the tests, was modelled using the package *glmmTMB* with beta distribution and logit link since is a proportion based on continuous data (Douma and Weedon 2019). As suggested in Douma and Weedon (2019), this initial model was compared to a model that allowed the dispersion parameter of the beta distribution to vary as function of treatment (using the function *dispformula*). This was done to account for potential heterogeneity in the dispersion of exploration among treatments (Douma and Weedon 2019). Model fit was then compared using the function AICtab in the package *bbmle.* The model that allowed the "precision" (dispersion) parameter of the beta distribution to vary across treatment groups, to account for heteroscedasticity, had considerably lower DeltaAIC and likelihood ratio test and is thus presented in results. In the model with dispersion parameter the interaction is not statistically significant and adding or removing the interaction does not affect the fit of the model and in the results is thus presented the simpler model without interaction. The dispersion model accounted for treatment-specific heterogeneity in behavioural variability, although residual diagnostics indicated some remaining deviation from the expected residual distribution. Offspring mean velocity and overall activity (also called total distance) in the open field were positively correlated (Pearson's $r = 0.67$, $P = <.001$). Therefore, only the model for overall mean velocity is presented in results (variable was log transformed to reach normality of residuals); the model for overall activity yielded quantitatively similar results (results not shown).

Critical swimming speed (hereafter swimming performance) was calculated using Brett's equation (Brett 1964) and it was adjusted for body length following methods in Nicoletto (1991). The model included treatment and brood size at 30 days old as covariates and female ID as random factor, to control for non-independence of data collected from siblings. The formula for critical swimming speed models an increment of water velocity every minute of the trial, whereas in this study the water

velocity increased up to the 5th minutes (when it reached 22 cm s^{-1}) and then it was maintained constant (see mehods above). Fifteen individuals (out of the 346 individuals for which swimming performance was measured) were swimming at maximum speed for more than a minute (6 control, 4 early and 5 offspring from late-exposed mothers). We thus ran the same model also without these potential outliers and the results were qualitatively the same (results not shown) and thus the full dataset was maintained for the analyses.

c) Adult offspring phenotype

Adult offspring body size was analyzed according to maternal treatment and models included density in the rearing tank (up to five individuals but some mortality occasionally occurred, see also above), as well as the sex of offspring (sons or daughters), since in this species females are substantially larger than males. The interaction between maternal treatment and sex of the offspring was initially included in the model for body size at sexual maturation, to investigate for sex-specifics effects on growth, but was removed because not significant ($P > 0.1$). Female ID (i.e. mother identity) was included as random factor to control for non-independence of data collected from siblings.

Adult males (i.e. sons) color patterns (proportion of orange and black coloration on body area and orange chroma, i.e. the proportion of orange light on the whole spectrum) were analysed using the package *glmmTMB* and beta distribution with logit link (Douma and Weedon 2019). The model also included maternal treatment and sex ratio (calculated as the proportion of males within each brood). Female ID (i.e. mother identity) was included as random factor to control for non-independence of data collected from siblings. As suggested in Douma and Weedon (2019) all three models were compared to a covariate dispersion model allowing dispersion parameters to vary according to treatment (using the function *dispformula*), as done in the models for proportion of arena explored, and then the models were compared using the function AICtab in the package *bbmle.* For proportion of orange on body area and for orange chroma, the best models (lower DeltaAIC) were the simpler model, whereas for proportion of black the best model (lower DeltaAIC) was the one considering a covariate dispersion model on treatment which also fit better the residuals. All models, with/without dispersal parameters gave equivalent results. Colour brightness was measured with a linear mixed model (*lmer*, gaussian distribution) including maternal treatment and sex ratio as well as female ID as random factor.

Sperm velocity (VCL) was analysed using *lmer* (gaussian distribution) with maternal treatment as fixed factor. Female ID was included as random factor to control for non-indipendence of data

collected from sibling. Sperm count was analysed with a similar model but the variable was log-transform to reach normality of the residuals and standard length of the male was also included as covariate to take into account any size-effects on sperm count. Sex ratio in the tank was also initially included in both models for velocity and sperm count, however, it was removed because not significant ($P > 0.1$, see above).

Results

Females' reproductive success

Among all the 116 females in the experiment, 81.4% of control females (CTRL 35 females out of 43), 82% of early-exposed females (HWE 32/39) and 85% of late-exposed females (HWL 29/34) gave birth. Overall, females across the three different treatment groups did not differ in standard length ($F = 0.643$, $df = 2$, $P = 0.528$). Females did not differ in the probability of giving birth across treatments ($X^2 = 0.230$, $df = 2$, $P = 0.891$) or according to body size ($X^2 = 0.102$, $df = 1$, $P = 0.750$). Heatwaves during pregnancy did not affect gestation duration ($F = 0.767$, $df = 2$, $P = 0.467$;) nor the number of offspring produced ($X^2 = 0.61$, $df = 2$, $P = 0.739$). Female size did not impact gestation duration ($F = 0.118$, $df = 1$, $P = 0.732$; Figure S1a, Table S2) but, as expected, larger females produced more offspring ($F = 34.869$, $df = 1$, $P = <0.001$; Figure S1b, Table S2).

Offspring mortality

The probability to have at least one dead offspring within the brood was significantly explained by the interaction between female body size and treatment (Table 1a). Large females exposed late during the pregnancy had higher probability of offspring mortality (slope analysis, HWL: $P = 0.03$), whereas no significant size-mediated relationships were detected in control or early-exposed females. Pairwise contrasts further indicated a higher probability of offspring mortality in late-exposed compared to control females (CTRL - HWL; $P = 0.021$), while early-exposed females showed a non-significant tendency (CTRL - HWE; $P = 0.086$) in the same direction (Table 1a and Figure 2).

When considering the proportion of live and dead offspring at 30 days, there was a non-significant trend ($P = 0.094$) in the interaction between treatment and body size (Table 1b). Large late-exposed females tended to have more dead offspring within their brood (slope analysis, HWL: $P = 0.057$) and pairwise contrast also suggested a tendency in higher proportion of dead offspring in late-exposed females compared to control (CTRL - HWL; $P = 0.084$; Table 1b, Figure 3).

Table 1: Output of GLM models on the probability to have at least one dead offspring (a) and on the proportion of dead/live young at 30 days old according to maternal treatment (3 groups: CTRL, HWE and HWL) and female body size. The table presents Anova results, slope analyses describing the relationship between female body size and response variables within each treatment, and pairwise contrasts among treatments. In bold values that are statistically significant ($P < 0.05$) and in italics values between $0.05 < P < 0.1$.

a) Probability of offspring mortality				
			X^2	df	P
	Female body size		2.544	1	0.111
	Treatment		9.648	2	**0.008**
	Female body size × Treatment		9.828	2	**0.007**
		Estimates ± SE	z		P
	Slope for length within CTRL females	-2.15 ± 1.41	-1.523		0.128
	Slope for length within HWE females	1.76 ± 1.19	1.478		0.140
	Slope for length within HWL females	4.12 ± 1.88	2.187		**0.029**
	Contrast		z		P
	CTRL - HWE		-2.117		*0.086*
	CTRL - HWL		-2.664		**0.021**
	HWE - HWL		-1.058		0.540
b) Proportion of live and dead offspring at 30 days				
			X^2	df	P
	Female body size		1.161	1	0.281
	Treatment		4.993	2	*0.082*
	Female body size × Treatment		4.738	2	*0.094*
		Estimates ± SE	z		P
	Slope for length within CTRL females	1.147 ± 1.064	1.078		0.281
	Slope for length within HWE females	-0.927 ± 0.862	-1.075		0.282
	Slope for length within HWL females	-2.176 ± 1.144	-1.902		*0.057*
	Contrast		z		P
	CTRL - HWE		1.513		0.285
	CTRL - HWL		2.128		*0.084*
	HWE - HWL		0.871		0.658

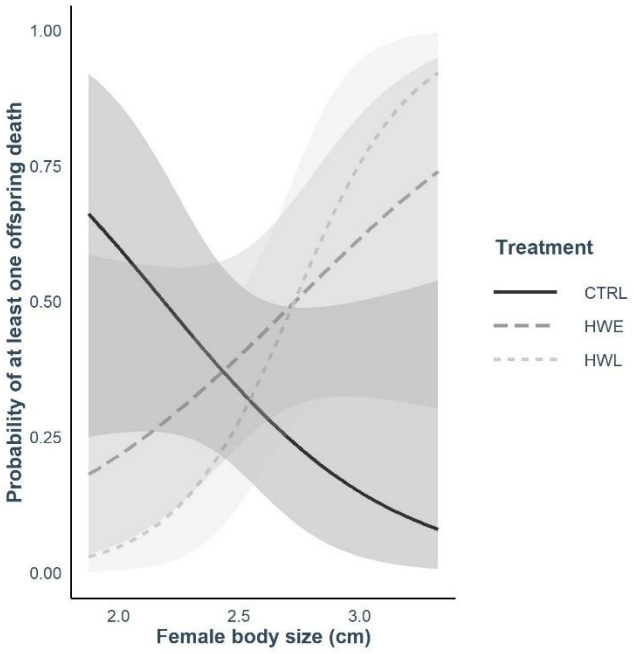

Figure 2: Estimates with CI of the glm model of the interaction between treatment (CTRL = control, solid line; HWE = early-exposed, dashed line; HWL = late-exposed females, dotted line) and female body size (standard length in cm) on the probability to have at least 1 offspring dead in the brood.

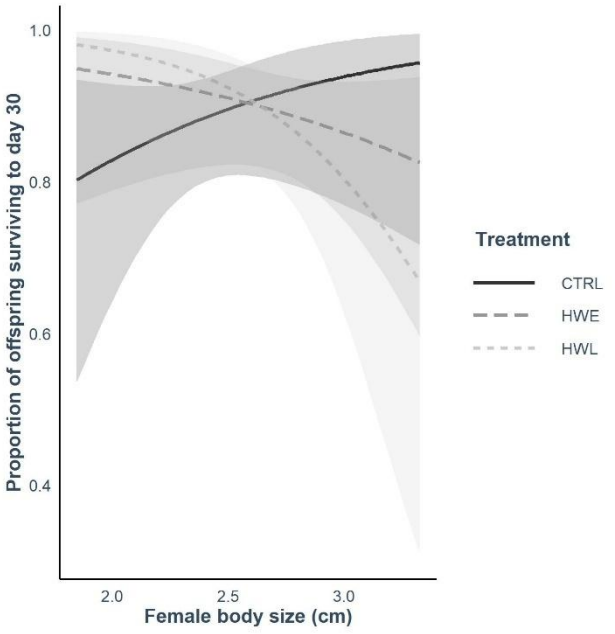

Figure 3: Estimates with CI of the glmmTMB model of the interaction between treatment (CTRL = control, solid line; HWE = early-exposed, dashed line; HWL = late-exposed females, dotted line) and female body size (standard length in cm) on the proportion of surviving offspring at 30 days old (i.e. proportion of dead/live offspring).

439 Juvenile offspring morphology

440 Offspring body size at 24 h was measured for 697 offspring from a total of 65 broods (Table S1).
441 Offspring body size at birth did not differ, neither according to maternal treatment ($X^2 = 0.009$, df =
442 2, P = 0.996) nor according to brood size ($X^2 = 1.38$, df = 1, P = 0.240). Offspring size at 30 days old
443 was measured in 680 offspring from a total of 63 broods (Table S1). Offspring size at 30 days did not
444 vary according to maternal treatment ($X^2 = 0.456$, df = 2, P = 0.796) but juveniles were smaller if
445 density was high during the first month of life ($X^2 = 87.90$, df = 1, P = <0.001).

446 Juvenile offspring behaviour and swimming performance

447 As expected, offspring explored less after exposure to conspecific alarm cues (Phase; Table 2a).
448 Maternal treatment also affected the proportion of the arena explored: offspring of late-exposed
449 females explored significantly more than offspring of early-exposed females and tended to explore
450 more than offspring of control females (Treatment; Table 2a, Figure 4).

451 In addition to these differences in mean exploration, the beta regression model allowing treatment-
452 specific dispersion indicated significant differences in behavioural variability among treatments.
453 Relative to controls, offspring of early-exposed females had significantly greater dispersion around
454 the estimated mean (dispersion estimate -0.314 ± 0.144, z = -2.18, P = 0.029), whereas offspring of
455 late-exposed females exhibited lower dispersion (dispersion estimate 0.352 ± 0.150, z = 2.34, P =
456 0.019). Thus, offspring of late-exposed females not only explored larger areas on average, but their
457 exploratory behaviour was also less variable at the treatment-group level than control offspring. In
458 contrast, offspring of early-exposed females did not vary in mean exploration from control but
459 exhibited greater behavioral variability. This pattern was also consistent with a significant Levene's
460 test for homogeneity of variance among treatments (Levene's test for homogeneity of variance for
461 treatment: F = 3.73, df = 2, P = 0.024).

462 After being exposed to the alarm cue, offspring were significantly slower compared to before
463 exposure (Phase; Table 2b) and swimming velocity in the open field also significantly varied
464 according to maternal treatment (Table 2b). Offspring of late-exposed females were significantly
465 faster than offspring of control females both before and after alarm cue (Table 2b and Figure 5).

466 Juveniles at 30 days old did not vary in their critical swimming speed, a proxy of swimming
467 performance and fatigue resistance (hereafter swimming performance), according to maternal
468 treatment ($X^2 = 2.913$, df = 2, P = 0.233). Swimming performance also did not differ according to the
469 density during growth (i.e. brood size at 30 days, $X^2 = 0.877$, df = 1, P 0.349).

Table 2: Anova and contrast analyses of the glmmTMB models on a) the proportion of arena explored both before-after alarm cues exposure and b) the lmer models for mean velocity (log-transformed) before-after alarm cues exposure. Model a) includes a covariate model for the dispersion parameter according to treatment (see results and statistical methods). In all the models: maternal treatment (control CTRL, early-exposed HWE and late-exposed HWL), phase (before PRE and after POST exposure to alarm cues) and brood size (at 30 days old) are included. The interaction Treatment × Phase was originally included in all the models but was removed because not significant (see *Statistical methods*). In bold values that are statistically significant ($P < 0.05$) and in italics values between.

a) Proportion of area explored

	X^2	df	P
Treatment	13.000	2	**0.002**
Phase	440.801	1	**<0.001**
Brood size	3.260	1	*0.071*
Contrast	z		P
CTRL - HWE	0.790		0.709
CTRL - HWL	-2.130		*0.084*
HWE - HWL	-3.069		**0.006**

b) Swimming velocity in the open field

	X^2	df	P
Treatment	8.096	2	**0.017**
Phase	14.234	1	**<0.001**
Brood size	0.509	1	0.476
Contrast	t		P
CTRL - HWE	-0.802		0.704
CTRL - HWL	-2.730		**0.023**
HWE - HWL	-2.065		0.109

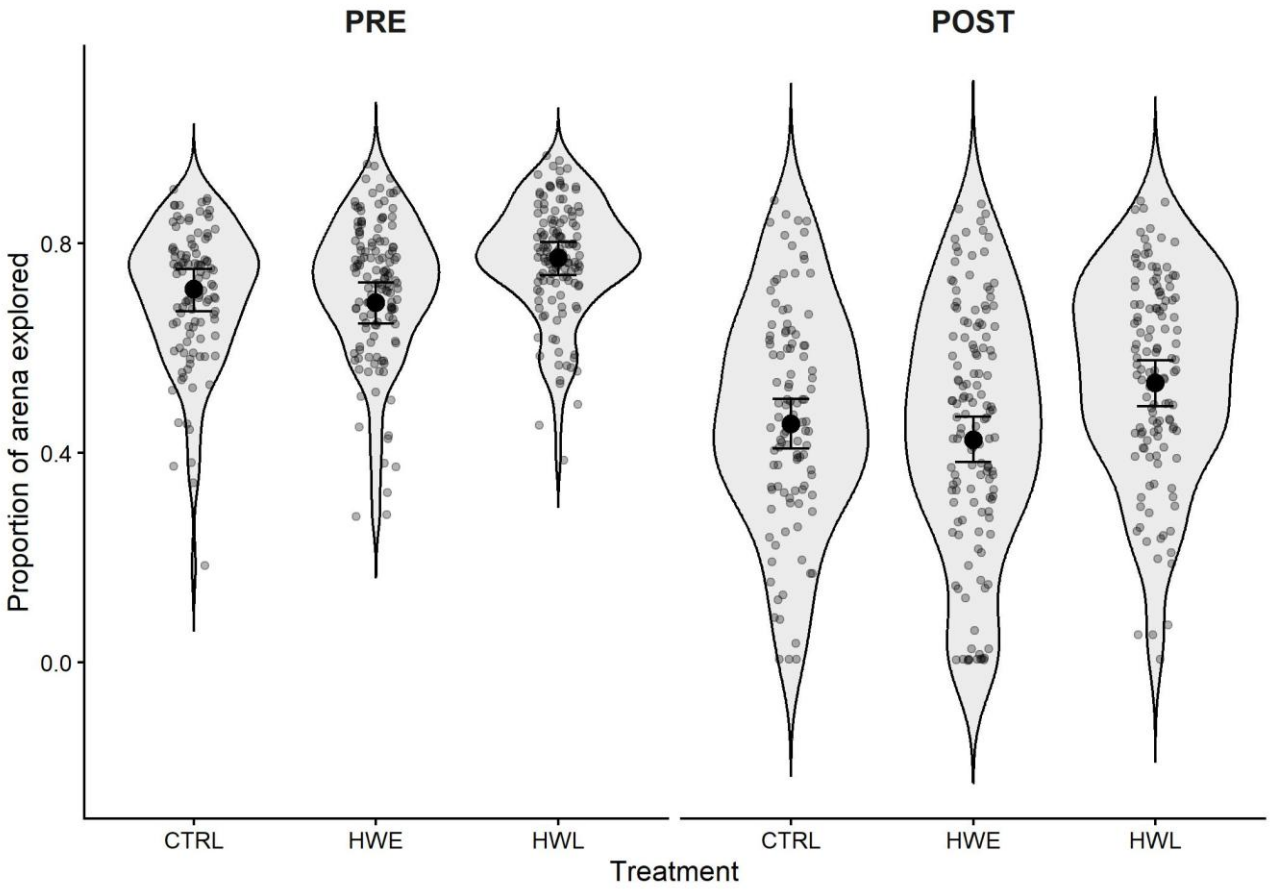

Figure 4: Estimated marginal means (± 95 % CI) from the glmmTMB model with treatment-specific dispersion and raw data showing the proportion of the arena explored by juveniles during open-field test according to maternal treatment (control CTRL, early-exposed HWE and late-exposed HWL) before (PRE) and after (POST) exposure to alarm cues.

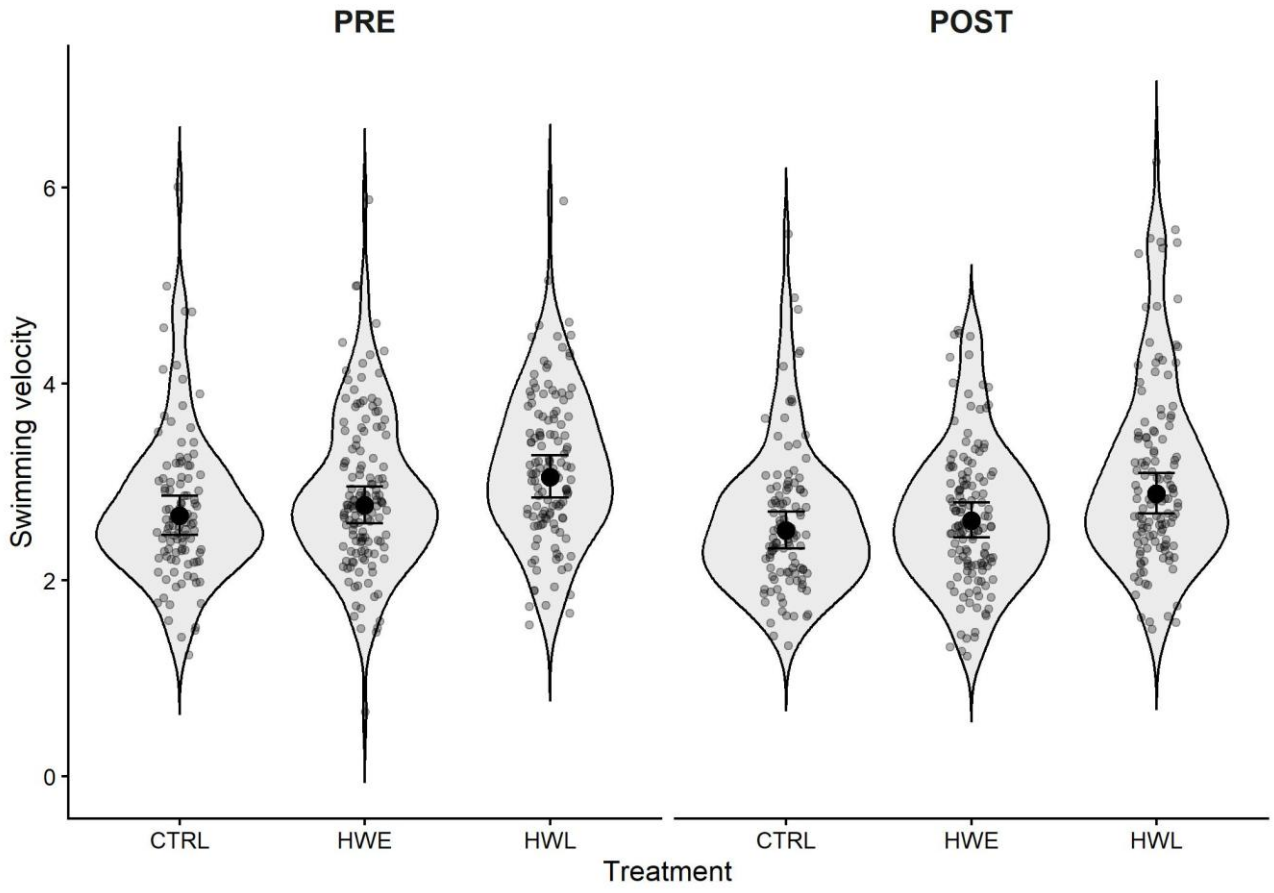

Figure 5: Estimated marginal means (± 95 % CI) from the linear mixed-effects model and raw data showing juvenile swimming velocity during the open-field test according to maternal treatment (control CTRL, early-exposed HWE and late-exposed HWL) before (PRE) and after (POST) exposure to alarm cues.

Adult phenotype

Among the 357 offspring, from overall 84 broods, that were raised to sexual maturation, 2 males (both from 2 distinct HWE families) were excluded from the analyses for size and sexually-selected traits due to abnormal size and complete lack of gonads (see supplementary materials).

Offspring size at adulthood was measured in a total of 355 offspring (see Table S1). Offspring size at adulthood did not depend on maternal treatment ($X^2 = 0.641$, df = 2, P = 0.726) but differed according to sex ($X^2 = 2380.099$, df = 1, P = <0.001), with daughters bigger than sons, and with overall offspring being smaller if they grew up in high density groups ($X^2 = 17.577$, df = 1, P = <0.001).

Adult offspring exhibited a different proportion of orange coloration depending on the timing of maternal exposure to heatwaves, whereas sex ratio during growth did not affect orange coloration

(Table 3a). Post-hoc tests showed that offspring of late-exposed females had a significantly higher proportion of orange coloration than offspring of early-exposed females, whereas neither treatments differed significantly from the control group (Table 3a; Figure 6). In contrast, black coloration did not differ in mean proportion among maternal treatments (Table 3b; Figure 7), but was affected by tank sex ratio, with males reared in a more male-biased groups exhibiting a greater proportion of black coloration relative to body size (sex ratio ± sd: 0.466 ± 0.19; Table 3b).

Despite the absence of treatment differences in mean black coloration, the dispersion model indicated significant differences in offspring phenotypic variability among treatment groups. Relative to controls, offspring of early-exposed females showed significantly greater dispersion around the estimated mean (dispersion estimates 0.766 ± 0.276, z = 2.77, P = 0.006). Offspring of late-exposed mothers also showed greater dispersion than controls (dispersion estimates 0.611 ± 0.312, z = 1.961, P = 0.050). Thus, maternal heatwave-exposure during both early and late pregnancy was associated with increased phenotypic variability in black coloration within treatment groups, despite having no detectable effect on the mean proportion of black coloration (Figure 7). This interpretation was further supported by a significant Levene's test for homogeneity of variance among treatments (F = 3.826, df = 2, P = 0.023). Finally, concerning color spectra measurements, neither brightness nor orange chroma differed according to maternal treatment or sex ratio (Table 3c, d).

Sperm velocity and count were estimated in 143 and 140 male offspring, respectively (Table S1). Neither trait differed according to maternal treatment (sperm velocity: $X^2 = 0.897$, df = 2, P = 0.639; sperm count $X^2 = 1.474$, df = 2, P = 0.479). Sperm count was also not significantly explained by male size ($X^2 = 0.455$, df = 1, P = 0.500).

Table 3: Male offspring coloration at sexual maturity. Anova and contrast analyses of glmmTMB models on the proportion of orange (a) and black (b) coloration on the total body area. Anova analyses for lmer model for brightness and (c) glmmTMB models for proportion of orange chroma. Model for proportion of black (b) includes a covariate model for the dispersion parameter according to treatment (see statistical methods and results). All models include female ID as random. In bold values that are statistically significant (P < 0.05) and in italics values between.

a) Proportion of orange coloration

	X^2	df	P
Treatment	6.168	2	**0.046**
Sex ratio	0.206	1	0.650

	Contrast	z		P
	CTRL - HWE	1.557		0.265
	CTRL - HWL	-1.041		0.551
	HWE - HWL	-2.450		**0.038**
b) Proportion of black coloration				
		X^2	df	P
	Treatment	3.484	2	0.175
	Sex ratio	5.842	**1**	**0.016**
	Contrast	z		**P**
	CTRL - HWE	1.863		0.150
	CTRL - HWL	1.065		0.536
	HWE - HWL	-0.693		0.768
c) Brightness				
		X^2	df	P
	Treatment	0.320	2	0.852
	Sex ratio	0.071	1	0.790
d) Orange Chroma				
		X^2	df	P
	Treatment	0.310	2	0.857
	Sex ratio	2.496	1	0.114

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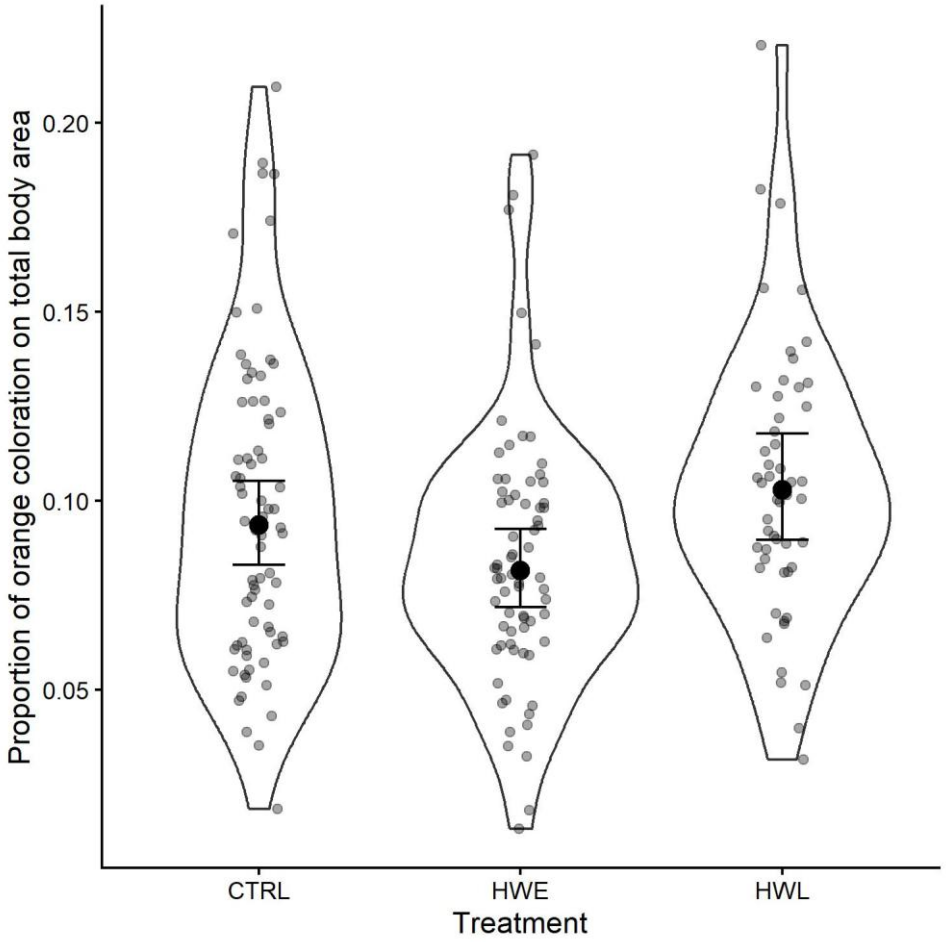
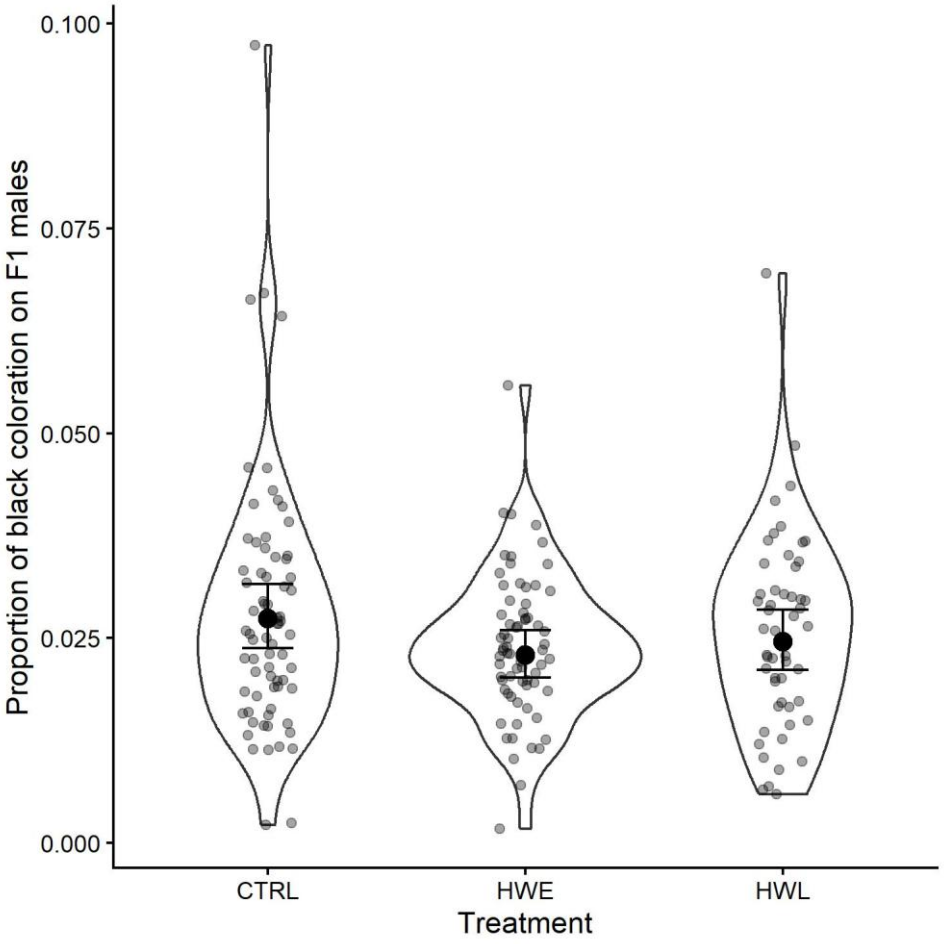


534

535 **Figure 6:** Estimated marginal means (± 95 % CI) from the glmmTMB model and raw data showing
536 the proportion of orange coloration relative to the total body area of adult offspring according to
537 maternal treatment (control CTRL, early-exposed HWE and late-exposed HWL).



538

539 **Figure 7:** Estimated marginal means (± 95 % CI) from the glmm TMB model with treatment-specific
540 dispersion and raw data showing the proportion of black coloration relative to the total body area in
541 adult offspring according to maternal treatment (control CTRL, early-exposed HWE and late-exposed
542 HWL).

543

Discussion

Timing of heatwave exposure during pregnancy emerged as a primary driver of increased offspring mortality and altered offspring phenotypes, particularly in terms of behavioural and sexually selected traits. Heatwaves did not produce a uniform disruption of offspring development, instead, the gestational stage at which females experienced thermal stress determined which offspring traits were affected. Late gestation likely represents a period of increased vulnerability because maternal energetic expenditure, embryonic oxygen demand and reductions in thermal tolerance all peak during this stage (Auer et al., 2021; Ghalambor et al., 2004). However, maternal heat stress during late gestation, as well as the possible direct effects of heat on growing embryo, did not cause a general developmental impairment in the offspring but only affected probability of offspring mortality in late-exposed females. Traits associated with somatic growth, swimming performance and sperm quality remained unaffected, whereas exploratory behaviour and male ornaments were altered. Moreover, for some traits maternal thermal stress altered phenotypic variability independently of mean trait values. In particular, behavioural exploration and male black coloration differed in their variability among treatment groups, suggesting that thermal prenatal stress may influence also the distribution of offspring phenotypes. Our results indicate that pregnancy should not be viewed as a homogeneous window of thermal susceptibility. Rather, the developmental timing of maternal heat exposure determines both the magnitude and the nature of maternal effects on offspring.

Heatwave exposure late in pregnancy revealed a size-dependent pattern in offspring mortality. Larger females exposed late had higher probability of within-brood mortality compared to smaller females, whereas female size was unrelated to offspring mortality in control and early-exposed females. Moreover, the overall survival of the offspring at 30 days of age depended on maternal treatment and mother size, with a non-significant tendency for large females exposed late to heatwaves to have higher number of dead offspring within their broods. These results suggest that exposure to heatwaves during late stages of pregnancy may change the size-dependent advantage of larger females, as suggested within a climate warming scenario (e.g. in live-bearing mosquitofish *Gambusia affinis*; Fryxell et al. 2020). While larger body size was indeed associated with greater reproductive output, late gestational heatwaves appeared to erode this advantage by increasing the probability of offspring mortality among large guppy females. Thus, the most successful breeders, the large females capable of producing numerous offspring per brood (Reznick & Endler, 1982; Magurran 2005), could have higher reproductive costs when exposed to heatwaves during a late phase of pregnancy. Although the underlying mechanism remains unknown, one possible explanation is that larger females experience greater oxygen limitation during late gestation, when both maternal metabolic demand and embryonic oxygen requirements are highest, but future investigations are essential to understand the mechanisms

behind these effects. The consequences of such size-biased effects may be particularly severe in species in which reproduction is skewed toward a subset of highly fertile individuals that contribute disproportionately to population recruitment (Morosinotto et al., 2025). By acting as abrupt, widespread extreme events whose frequency and intensity are rapidly increasing, heatwaves can simultaneously impact large numbers of individuals across multiple life and reproductive stages.

Surprisingly, heatwave exposure during pregnancy did not affect offspring size, neither at 24 h after birth nor at juvenile or adult stage. Reductions in offspring size following maternal or embryonic exposure to heatwaves have been previously reported across multiple taxa (reviewed in Morosinotto et al., 2025), including fish (e.g. Alvarez-Quintero et al. 2025). However, when heatwaves are mediated by the parents' offspring size may not be affected even though offspring may be in worse body condition (Barrett & Stein, 2024). In this study, somatic growth was not impaired by maternal heat stress, whereas behavioural and other phenotypic traits exhibited a more plastic response.

Heat exposure during late gestation led to increased exploratory behaviour and higher average speed in the open field, both before and after exposure to conspecific alarm cues. Across all maternal treatments, juveniles exhibited antipredator responses, becoming less explorative and slower after alarm cues exposure. However, offspring of mothers exposed late in pregnancy remained consistently more exploratory and active than all other groups, also after exposure to alarm cues. A similar pattern was observed also in juvenile guppies directly exposed to heatwaves at one month of age, which moved greater distances than controls (Breedveld et al., 2025). Similar behavioural changes have previously been reported following maternal exposure to non-thermal stressors during pregnancy, including simulated predation, in both guppies (Cattelan et al., 2020; Stein and Hoke 2022) and mosquitofish *(Gambusia affinis;* McGhee et al., 2021). Increased explorative behaviour can be associated with dispersal propensity (Cote et al., 2010) and prenatal stress may have long-lasting effects on offspring explorative behaviour, carrying on until adulthood (McGhee et al., 2021). Maternal stress may thus alter offspring propensity to explore and disperse, potentially via embryonic exposure to maternal hormones. For example, in birds, increased exposure to maternal androgens has been linked to increased propensity to disperse (Tschirren et al., 2007). Notably, offspring of late-exposed mothers exhibited reduced within-treatment behavioural variability. On the other hand, offspring of early exposed mothers, despite not altering mean exploratory behaviour, appeared to have greater within-treatment variation, suggesting a wider distribution of behavioural phenotypes. Thus, maternally mediated stress during late gestation could produce a consistently more exploratory phenotype, whereas early exposure could lead to increased phenotypic variability, potentially producing individuals with wider ranges of behavioral responses.

An altered exploration pattern could also result from a direct, passive, effect of maternal stress to the offspring and be linked to morphological or physiological conditions of the offspring themselves. However, in this study the exploratory behaviour of the young guppies was not linked to condition, since there were no differences in offspring swimming performance nor in body size at the time of the observations in the open field test. The absence of treatment differences in swimming performance suggests that increased exploration was unlikely to result from differences in locomotor capacity or fatigue resistance. However, increased exploratory behaviour should not necessarily be interpreted as an adaptive response. It could also reflect stress-induced hyperactivity or altered anxiety-like behaviour (Yeramilli et al. 2024) driven by the stress perceived by the mother during gestation. Alternatively, the differences in explorative propensity and velocity in open field observed in offspring of late-exposed mothers, could also be the result of differential mortality within the broods, with only some offspring phenotypes surviving the heatwave. Nevertheless, the lack of differences in swimming performance or body condition (body size), and the behavioural differences persisting both before and after exposure to conspecific alarm cues, seems consistent with a stable behavioural phenotype rather than a transient stress response. Further studies should investigate within and among individual variation under heat stress as this could affect the potential of individuals to adapt to future conditions.

Adult offspring of mothers exposed to heatwaves during late gestation exhibited a higher proportion of orange coloration at sexual maturation compared to offspring of early-exposed mothers, whereas brightness and orange chroma did not differ across treatments. In male guppies, orange coloration is a sexually selected trait strongly preferred by females, and individuals exhibiting a larger proportion of orange relative to body size achieve higher reproductive success (Magurran 2005). Thus, heat stress endured during late gestation resulted in a sexual trait that appears to provide higher fitness benefits in adulthood. This pattern may reflect the elevated within-brood mortality among large females exposed to heatwaves late in pregnancy. If differential mortality operates during embryonic development, surviving offspring of late-exposed females may represent individuals in better condition or of different genotypes, capable of producing larger sexual ornaments once sexually mature. It is important to note, however, that even through offspring of late exposed mothers were more orange than those of early-exposed mothers, their orange levels were not significantly higher than those in the offspring of control females. Thus, offspring of late-exposed mothers would not necessarily have a substantially higher mating success respect to offspring of control females.

In contrast to orange coloration, black coloration in offspring did not exhibit clear maternal effects in terms of mean expression. However, despite the absence of differences in mean expression, offspring of early-exposed females, and to a lesser extent also those of late-exposed females, displayed

significantly greater within-treatment variability in the proportion of black coloration. This suggests that, although maternal treatment did not shift average amount of black ornamentation, heatwave exposure during both early and late gestation increased phenotypic variability. Very little is currently known on how maternal or early-life stress influences the development of pre-copulatory sex traits. In guppy, juveniles exposed to predator cues early in life exhibit altered coloration patterns (Ruell et al., 2013). In another Poeciilid fish (*Poecilia wingei*), thyroid hormones, which are functionally linked to cortisol dynamics, regulate the development of black melanic spots (Prazdniko 2021). Future studies should thus investigate if maternal stress could indirectly influence embryonic hormonal levels potentially affecting multiple downstream developmental pathways, linked to both black and orange ornaments. Despite these observed differences on pre-copulatory sexual traits, no apparent patterns appeared on post-copulatory sex traits, with no differences in sperm velocity and counts according to maternal treatment.

Heatwaves reduced female reproductive success and, importantly, induced maternal effects that shaped offspring size and behaviour, with consequences persisting into adulthood and affecting fitness-related sexual traits. Viviparous vertebrates are particularly susceptible to glucocorticoid-mediated maternal effects due to the prolonged physiological connection between the female and developing embryo (MacLeod et al 2021). Even in live-bearing lecitothropic species, such as the guppy, where embryonic nutrients are largely derived from yolk reserves, stress-mediated maternal effects can arise because oxygen exchange and excretion occur through exchange with the maternal environment (Uribe and Grier 2010). Moreover, in live bearing species, maternal physiology also mediated the thermal environment experienced by developing embryos. Unlike oviparous embryos, that are directly exposed to environmental temperatures, in live-bearing species embryos thermal experience depends on maternal exposure. Thus, heatwaves may affect developing embryo but the magnitude and consequences of this exposure remain partly mediated by maternal physiology. These characteristics make live-bearing species particularly valuable for investigating how heatwave-induced maternal stress influences offspring development and whether such effects persist across generations.

Females exposed to heatwaves during late gestation produced offspring with greater exploratory propensity and enlarged orange sexual ornaments, traits that can both have profound ecological and evolutionary consequences. Interestingly, maternal heat stress, potentially combined with maternally-mediated embryonic heat stress, affected behavioural and sexually selected traits while leaving offspring growth, swimming performance and sperm traits unchanged. This suggests that maternally-mediated thermal stress does not induce generalized developmental impairment, but instead affects a subset of traits that may be particularly sensitive to developmental programming. Together, these

679 effects are consistent with the presence of heatwave-mediated transgenerational effects on both
680 behavioural and sexually selected traits. These results highlight that the developmental stage at which
681 extreme temperatures occur determines whether maternal stress is translated into persistent changes
682 in offspring phenotype. The timing of heatwaves during reproduction therefore emerges as a crucial
683 determinant not only of embryo survival, but especially of how environmental stress shapes offspring
684 phenotypes with persisting effects into adulthood and potentially influencing subsequent generations.
685 Understanding the extent to which heatwaves generate transgenerational effects in natural populations
686 is essential, as such cryptic effects may influence key ecological and evolutionary processes, from
687 dispersal dynamics and antipredator strategies to sexual selection mechanisms, by reshaping
688 behavioural and ornamental traits across generations.

689

690 ***Authors contribution***

691 Chiara Morosinotto, Clelia Gasparini and Merel Breedveld conceived the ideas and designed
692 methodology; Chiara Morosinotto, Alessandra Stevanato, Nadia Zanelli and Alessandro Devigili
693 collected the data; Chiara Morosinotto and Merel Breedveld analysed the data; Chiara Morosinotto
694 led the writing of the manuscript. All authors contributed critically to the drafts and gave final
695 approval for publication.

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707 European Commission and ERCEA. Analyses of behavioural videos were conducted with the help of
708 the Behavioral phenotyping facility at Department of Biology, University of Padova.

709 ***Ethics Statement***

710 All experiments were carried out with the approval of the University of Padova's Animal Ethics
711 Committee and the national authorities; Organismo Preposto al Benessere degli Animali (OPBA;
712 approval no: 313/2022-PR).

713

Supplementary materials

Table S1: Sample size throughout the experiment. In a) number of females that entered the experiment, that gave birth and for which we have information on gestation duration. In b) the sample size for all the offspring traits included in the manuscript. *Total broods* correspond to the total number of broods produced and *total offspring* comprehend the whole offspring sample size, all broods and individuals for which we could collect data on offspring phenotype (numbers in parenthesis correspond to the number of broods included in each analysis). Each female produced one brood within this dataset.

a)

Trait	Experimental unit	Control	Early-exposed	Late-exposed	Total individuals
Females entering experiment	Females	43	39	34	116
Females giving birth	Females	35	32	29	96
Gestation duration	Females	32	32	28	92

Note: the number of females considered gestation duration is 92 while total broods produced is 91 because one female died during delivery, thus she is included among the females that gave birth and gestation duration but could not be considered for total offspring or any other offspring phenotype analyzes as total number of offspring was not known.

b)

Trait	Experimental unit	Control (n.broods)	Early-exposed (n.broods)	Late-exposed (n.broods)	Total individuals	Total broods considered
Total broods	Brood	31	32	28	-	91
Total offspring	Brood	343(31)	441(32)	367(28)	1151	91
No. offspring size at 24 h	Offspring	207(23)	256 (21)	234 (21)	697	65
No. offspring size at 30 days	Offspring	189 (22)	248 (20)	242 (21)	680	63
No. offspring open-field behaviour	Offspring	106 (18)	136 (19)	127 (18)	369	55
No. offspring swimming performance	Offspring	113 (19)	105 (16)	128 (18)	346	53
No. female offspring adult size	Female offspring	55 (31)	52 (28)	53 (25)	160	84
No. male offspring adult size	Male offspring	73 (31)	70 (28)	52 (25)	195	84

No. male offspring orange color	Male offspring	73 (27)	70 (27)	52 (22)	195	76
No. male offspring black color	Male offspring	72 (27)	70 (26)	52 (22)	194	75
No. male offspring color spectrum	Male offspring	64 (25)	43 (17)	28 (13)	135	55
No. male offspring sperm velocity	Male offspring	44 (17)	50 (20)	49 (21)	143	58
No. male offspring sperm count	Male offspring	48 (18)	46 (20)	46 (21)	140	59

730

731 **Table S2**: Mean ± S.E. and range for gestation duration (days from T0 to delivery) and brood size at
732 24 h (number of offspring produced)

	Mean ± S.E.	Range
Gestation duration	24.467 ± 0.312	17-34
Brood size at 24 h	12.684 ± 0.808	1-34

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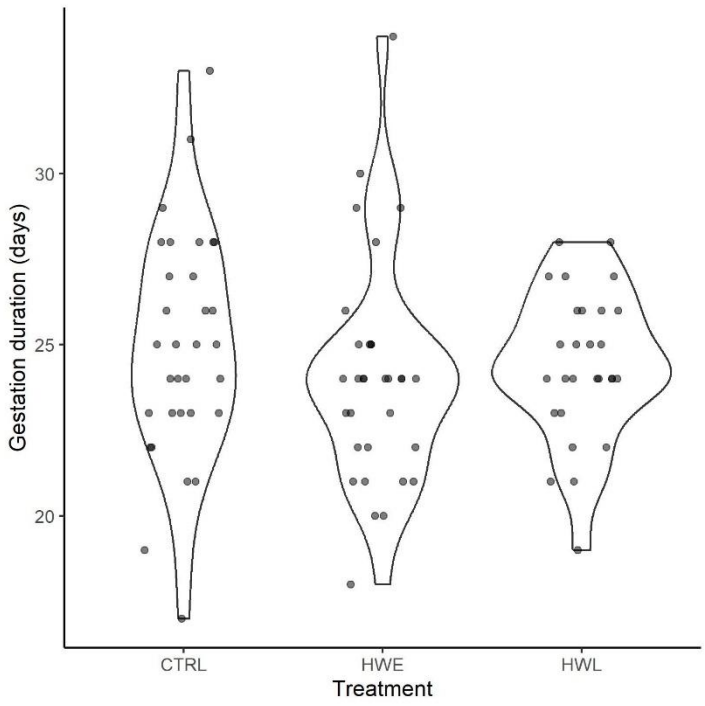
736

737 **Figure S1**: a) Gestation duration, as number of days necessary to delivery from T0, according to
738 treatment and b) brood size at 24 h according to treatment (CTRL=control females, HWE= early-
739 exposed females, HWL= late-exposed females).



740 a)

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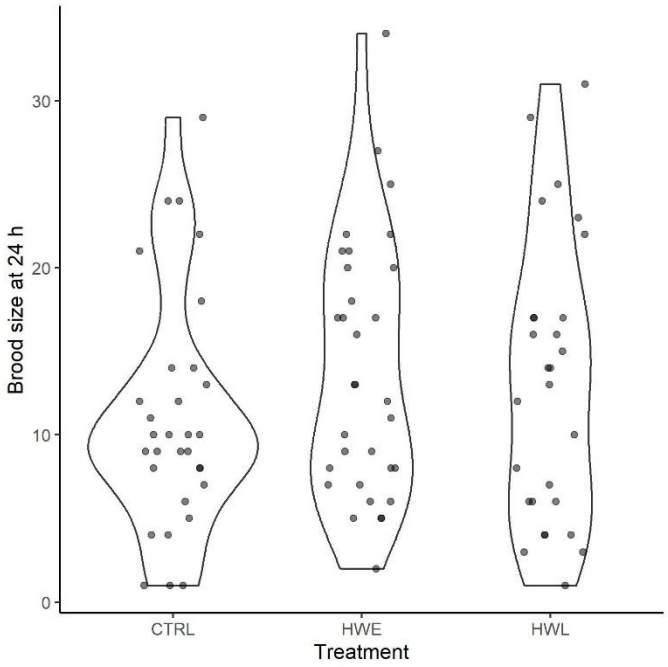

b)

<u>Offspring sexual maturation- 2 outliers</u>

Among the 357 offspring from overall 84 families that were brought to complete sexual maturation, 2 males (both from 2 distinct HWE families) were excluded from the analyses for size and sexually-selected traits due to abnormal size and lack of gonads. These two males appeared of abnormal size (both larger than 2 cm) and they did no present normal mature sexual coloration nor did they produce any sperms. Following this observation, upon anatomic examination, it was noticed that they completely lacked gonads. The female offspring from these two families where within the normal range of size for the species and were thus included in the analyses for size but upon anatomical examination it appeared that also several (but not all) of these female offspring had no, or limited, gonads.

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