Maternal temperature exposure triggers emotional and cognitive disorders and dysregulation of neurodevelopment genes in fish

Violaine Colson [Corresp., 1], **Morgane Cousture** [1], **Danielle Zanerato-Damasceno** [1], **Claudiane Valotaire** [1], **Thaovi Nguyen** [1], **Aurélie Le Cam** [1], **Julien Bobe** [Corresp. 1]

[1] Fish Physiology and Genomics, INRA LPGP UR1037, Rennes, France

Corresponding Authors: Violaine Colson, Julien Bobe
Email address: violaine.colson@inra.fr, julien.bobe@inra.fr

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Violaine Colson[1], Morgane Cousture, Danielle Zanerato-Damasceno, Claudiane Valotaire, Thaovi Nguyen, Aurélie Le Cam, Julien Bobe

INRA, UR1037 Fish Physiology and Genomics (LPGP), F-35000 Rennes, France

Corresponding Author:

Violaine Colson[1]

Email address: violaine.colson@inra.fr

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Abstract

Fish are sensitive to temperature, but the intergenerational consequences of maternal exposure to high temperature on offspring adaptive behaviour and underlying mechanisms are unknown. Here we show that a thermal maternal stress induces emotional and cognitive disorders in offspring. Thermal stress in mothers triggered the inhibition of fear responses and decreased spatial learning abilities in progeny. Impaired behavioural phenotypes were associated with the dysregulation of several genes known to play major roles in neurodevelopment, including *auts2*, a key gene for neurodevelopment, more specifically neuronal migration and neurite extension, and critical for the acquisition of neurocognitive function. In addition, our analysis revealed the dysregulation of another neurodevelopment gene (*dpysl5*) as well as genes associated with human cognitive disorders (*arv1*, *plp2*). We observed major differences in maternal mRNA abundance in the eggs following maternal exposure to high temperature indicating that some of the observed intergenerational effects are mediated by maternally-inherited mRNAs accumulated in the egg. Together, our observations shed new light on the intergenerational determinism of fish behaviour and associated underlying mechanisms. They also stress the importance of maternal history on fish adaptive capacities in a context of global climate changes.

Introduction

In the current context of global climate warming, wild and aquaculture fish are exposed to varying environmental factors including suboptimal temperatures at specific periods of their lifecycle. Fish are highly sensitive to extreme or abnormal (i.e. outside of the normal physiological range) temperatures throughout their lifecycle, even for short periods of time. This is especially true for key periods such as the reproductive period, during which the female gamete undergoes final oocyte maturation. The direct impact on gamete quality has been thoroughly investigated in many temperate species (see (Bobe & Labbe 2010; Kjorsvik et al. 1990; Migaud et al. 2013) for review). Exposure of mature female fish to high temperature during reproductive season (i.e. prior or around the time of ovulation) has a dramatic impact on egg size (Jonsson & Jonsson 2016), egg viability and subsequent embryonic success (Aegerter & Jalabert 2004) including reduced survival throughout development. Despite this well documented negative impact on egg quality and subsequent embryonic development, the long-term effects of maternal exposure to suboptimal temperature on progeny behaviour and adaptive capacities remain unknown. More specifically, the intergenerational consequences of mother exposure to abnormal temperature on offspring emotional responses and cognitive performances – two key components of fish adaptation and welfare – have never been investigated.

Several studies have however shown that maternal history can impact offspring behaviour and adaptive capacities (i.e. ability of an organism to change its morphology, physiology, or behaviour according to stressful environmental conditions (Bijlsma & Loeschcke 2005)). This intergenerational effect on offspring behaviour was observed in salmonid fish in which stress during reproductive season, or at least artificial exposure to stress hormones,

54 has a significant intergenerational impact on offspring adaptive capacities, including
55 modifications of cognitive abilities (Sloman 2010) and emotional reactivity (Colson et al.
56 2015b; Eriksen et al. 2011; Espmark et al. 2008). In contrast, the underlying mechanisms
57 mediating these effects remain poorly documented. In mammals, profound long lasting
58 behavioural deficits have been observed in mice originating from stressed mothers,
59 possibly due to epigenetic modifications occurring in the mother and transmitted to
60 offspring (Weiss et al. 2011). In fish, a recent study has demonstrated the existence of the
61 programming of stress axis function in zebrafish (*Danio rerio*) offspring by maternal social
62 status (Jeffrey & Gilmour 2016). Another study showed that three-spined stickleback
63 (*Gasterosteus aculeatus*) embryos respond to maternal exposure to predation risk via
64 changes in gene expression (Mommer & Bell 2014).
65 The aim of this study was to thoroughly characterize the impact of high temperature
66 exposure of female rainbow trout (*Oncorhynchus mykiss*) during reproductive season on
67 offspring emotional and cognitive phenotypes, using specific behavioural tests previously
68 validated in the laboratory (Colson et al. 2015a; Poisson et al. 2017; Sadoul et al. 2016). We
69 also aimed at deciphering the molecular mechanisms mediating such intergenerational
70 effects by analysing genome-wide gene expression in eggs and developing embryos
71 following maternal exposure to high temperature.
72

Material and methods

Ethics statement

Fish were reared in INRA LPGP facilities, which hold full approval for animal experimentation (C35-238-6). All fish were reared and handled in strict accordance with French and European policies and guidelines of the INRA LPGP Institutional Animal Care and Use Committee, which specifically approved this study (n° T-2016-55-VC-CV).

Maternal treatment and fertilization

Two-year old females rainbow trout were exposed to either 12°C (12°C group, standard reproduction conditions) or 17°C (17°C group, high suboptimal temperature) for six weeks before ovulation. The temperature of 17°C was selected because it is known to induce a dramatic decrease in embryonic survival (Aegerter & Jalabert 2004). For each group, 30 marked (external tag placed on the dorsal fin) females were kept in 2.5 m^3 tanks (2 x 2 x 0.62 m, length × width × water height). In the 17°C group, females initially reared at 12°C were acclimated for five days to an increase of 1°C/day until 17°C. For three weeks preceding ovulation, females were checked every two-three days to detect ovulation. In both experimental group, eggs originating from four simultaneously ovulating females of each group were collected and fertilized using a pool of sperm collected from four males held at 12°C. Fertilization was performed immediately in both groups in order to avoid any bias on subsequent behavioural phenotypes that would have been induced by differences during embryo development. For each of the eight females, fertilizations of 800 eggs were performed at 10°C using the medium ActiFish (IMV, L'Aigle, France; 100 ml ActiFish + 400 ml water) and fertilized eggs were distributed within a tray (20 x 50 cm) in two incubators

(10 x 10 cm) (approximately 400 eggs/incubator and two incubators/tray) supplied with 10°C flow-though recycled water. Each tray was covered with a lid to avoid exposure to light.

Monitoring of developmental success

Developmental success was monitored at eyeing stage, i.e. 19 days post-fertilization (dpf), hatching (32-33 dpf), and completion of yolk-sac resorption (YSR, 55 dpf) by counting dead embryos that were removed from incubators. The occurrence of malformations was obtained by taking a picture of euthanized malformed fry in each incubator at YSR. The types of malformed fry observed in this study were: torsion (T), yolk sac resorption defects (YSD) and other malformations (O) as described in (Bonnet et al. 2007). For each female, the occurrence of each type of malformations was calculated in comparison to the total number of malformed fry. Percentages of mortalities and malformations per incubator were obtained by counting the final number of live fry at swim-up stage, before transfer into rearing tanks.

Sample collection during embryo development

In both experimental groups, and in all egg clutches, biological samples were collected at four different stages: unfertilized eggs, around zygotic genome activation (i.e. 5 days post fertilization), hatching after removing yolk-sac, and YSR, which also corresponded to the stage of behavioural phenotyping. Entire (i.e. whole body) embryos were sampled. All samples were frozen in liquid nitrogen and held at -80°C until further processing.

Fry rearing

After vitellus resorption, at 55 dpf, swim-up fry from the two incubators per female were combined and transferred to seven distinct tanks (50 x 60 x 28 cm) (approximately 200 fry/84 L), corresponding to seven different females (four from the 12°C group and three from the 17°C group). The mortality rate of one of the 17°C female was 98.4% and we did not obtain enough offspring to perform behavioural phenotyping. For this female, we only sampled the remaining fry to perform transcriptome analyses. Water temperature was maintained at 12°C. Fry received manually four meals per day with a commercial diet (Biomar, 48% protein and 22% lipid, 0.5 mm diameter pellets). Tanks were automatically illuminated from 8:00 to 20:00. Before each behavioural test, fish were starved for 24h. At the end of each test, fish were netted and transferred into individual bowls containing 250 ml of the circuit water to which a lethal dose of anaesthetic (tricaïne: 4.5 ml + bicarbonate of sodium: 5 ml) had been added.

Phenotyping of offspring behaviour

For each female (three 17°C females and four 12°C females), different hatchlings were subjected to the following behavioural tests thoroughly described in (Poisson et al. 2017):

Assessment of offspring emotional reactivity:

Fish propensity to express adapted fear-related behaviour (e.g. emotional reactivity) was evaluated individually in a novel-tank test at 75-76 dpf (social isolation in a novel tank). The novel tank (30 x 19 x 16 cm) was supplied with 12°C flow-though recycled water. Fifteen fish per female were observed. The treatment order was randomly chosen.

Behavioural responses were video-recorded for 30 minutes, divided into six 5-min intervals and analysed with EthovisionXT software (Noldus, Netherland). The following behavioural parameters were calculated for each individual: total distance travelled (cm), maximum swimming velocity (cm/sec), angular velocity (°/sec) (i.e. erratic swim), and time spent (%) in the border zone (i.e. thigmotaxis). At the end of the test, each fish was measured and weighed.

Assessment of offspring spatial learning abilities and memory:

Offspring propensity to locate a food-rewarded arm was assessed in a T-maze supplied with 12°C flow-though recycled water (see (Poisson et al. 2017) for a complete setup description). Five fish per female were tested. After 24h food deprivation and 30min of acclimation in the start-box of the T-maze, a remote-controlled guillotine door was pulled-up and fish occupation of the maze was video-recorded for a maximum of 1800 seconds per trial. A visual cue (black cross) was located on the wall of the T-maze at the entrance of the reward arm. When the fish crossed an invisible line separating the rewarded arm from the rest of the maze, a mechanic ridge, remotely-controlled by an experimenter observing live videos in an adjacent control room, released pellets. Then the fish was left to eat the pellets for at least 5min before being gently netted and introduced in its individual holding tank until the next trial. Eleven successive trials were run for four consecutive days (two trials on the first day and three on the other days). The treatment order was randomly chosen on the first day. We measured the latency to leave the start-box (Latency SB) and the latency to reach the reward arm after the fish had left the start-box (e.g. right choice, Latency RC). Retention of the acquired information (i.e. memory) was evaluated three days after the last

158 trial by another trial (resulting in a 2-day break). We measured Latency SB and latencies to
159 make either the right (Latency RC) or the wrong choice (Latency WC).

160 Egg cortisol contain

161 Six eggs per female were homogenized in 600mL of deionized water using Precellys
162 Evolution (Bertin Technologies, France). The program used was: 2mL CK14 tubes (work
163 4x20s 6800rpm + 30s break). Extraction of cortisol was performed using 200µL of
164 homogenate (after short centrifugation at 3000g) and adding 2 mL of
165 ethylacetate/cyclohexane (50/50, vol/vol) at room temperature. The supernatant was
166 recuperated after strong mix and freezing at -20°C for at least 1 hour followed by another
167 round of ethylacetate/cyclohexane extraction. After solvent evaporation, extracted cortisol
168 was re-dissolved in 100µl ethanol. After evaporation, the dry residue was dissolved in
169 500µL buffer from cortisol ELISA kit purchased from Cayman Chemical (USA). Cortisol
170 levels were determined following the ELISA kit manufacturer's instructions. The
171 absorbance of each well was measured at 412 nm using a Synergy-2 microplate reader
172 from BioTek (USA) instruments. Cortisol levels of eggs were calculated based on the
173 calibration curve of absorbance. The assay has a range from 6.6-4000 pg/mL and a
174 sensitivity (80% B/B0) of approximately 35 pg/mL.

175 Gene expression profiling

176 Transcriptome analysis was conducted using four egg batches originating from female held
177 at 12°C and four egg batches originating from females held at 17°C, with the exception of
178 YSR/12°C for which only three RNA samples of sufficient quality could be obtained. RNA
179 was extracted from 20 eggs sampled at fertilization, 20 eggs at 5 dpf, six embryos at

hatching and six alevins sampled at YSR. Frozen tissues were lysed with Precellys Evolution Homogenizer (Ozyme, bertin technologies) in TRI Reagent (TR118, Euromedex) and total RNA was extracted according to the reagent's method followed by Nucleospin RNA isolation kit (740955, Macherey Nagel). Gene expression profiling was conducted using an Agilent 8x60K microarray (GPL24910) as previously described (Żarski et al. 2017). Samples were randomly distributed on the microarray for hybridization. The data were processed using the GeneSpring software (Agilent v.14.5) using gMedianSignal values. After data processing, one sample from the hatching/17°C group, which behaved differently form the other samples, even after normalization, was removed from subsequent analysis. Corresponding data were deposited in Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) under the reference GSE113377.

Statistics

Due to low number of synchronized females per treatment, percentage mortalities and malformations were compared between treatments using nonparametric Mann-Whitney tests (R, Mann-Whitney-Wilcoxon non-paired tests).

Fish weights were analysed after taking into account the temperature as a fixed factor (two levels: 12°C and 17°C) and the females as a random factor. A generalized linear mixed model (GLMM) was fitted using the nlme package in R 3.3.1 (http://cran.r-project.org/), and by assuming a normal distribution. Significance of the random effect was checked using the 95% confidence interval of the variance, 0 being excluded of the interval in case of significance.

The analyses of the novel tank test consisted in testing the effect of the temperature, the effect of the interval (two levels: 0-5 min and 25-30 min) and their interaction on each

dependant variable. The females and individuals (repeated measures) tested within treatments and intervals were defined as random factors in our statistical modelling. Distance travelled and maximum velocity were square root transformed, while angular velocity was log-transformed in order to reach normality and to fix GLMMs models using the nlme package. Significance of the random effect was checked using the 95% confidence interval of the variance, 0 being excluded of the interval in case of significance. When models were significant, post-hoc analyses were performed using HSD-Tukey tests. For thigmotaxis, data were too far from a normal distribution so we fixed a GLMM using the lme4 package, assuming a gamma distribution with inverse function. With this R package, a low variance associated with the random factor female indicated non-significant random effects.

The analyses of the spatial learning test consisted in testing the effects of the temperature, the trial (considered as a fixed factor with 11 levels, which are dependant data throughout the time), and their interaction on each dependant variable. The female and individuals (repeated measures) were defined as random factors. We fitted a GLMM model (using the lme4 package), assuming a normal distribution for Latency SB (after log transformation). Latency RC data did not reach normal distributions so we fitted a GLMM assuming a gamma distribution with inverse function. We fitted the same models for memory data considering the fixed factor trial with two levels (trial 11 vs. three days later). For latencies to make either the right or the wrong choice three days after the 11^{th} trial, we also fixed a GLMM model (gamma distribution with inverse function) to test the effects of the temperature, the entry choice (Latencies RC and WC) made by the fish (fixed factor with

two levels, which are dependent data), and their interaction. When models were significant, the summary was considered for pairwise comparisons.

For all models, if there were non-significant effects on factors or interactions, stepwise backward eliminations were performed to sequentially simplify the full model. The models were validated using analysis of residuals: normality assessment.

Egg cortisol contains were compared between treatments using nonparametric Mann-Whitney tests.

Differences were found to be significant when $P < 0.05$ and tendencies were considered for $0.05 < P < 0.1$. We indicated results of post-hoc analyses by different letters or by daggers symbols in the figures but they are not described in the Results section.

For microarray analysis, gene expression data was scale normalized and log(2) transformed before statistical analysis. The differences between the groups were analyzed using a two-way ANOVA with two factors (temperature, stage and their interaction), with a corrected *P*-value < 0.05 after Benjamini-Hochberg correction. For the four individual genes presented in Fig. 4C, non-parametric Mann-Whitney tests were performed between 12°C and 17°C groups within egg and 5 dpf stages to reveal any significant differential expression.

Results

Influence of maternal exposure to high temperature on developmental success and growth

Maternal exposure to high temperature had a major impact on offspring survival. A dramatic increase in mortality was observed throughout early development when eggs originated from females held at 17°C even though this difference was not significant until hatching due to a high variability (Fig. 1). The overall median (quartiles: 25 and 75%) mortality rate was below 10% in the 12°C group, while it was over 40% in the 17°C group, with 6.62(5.30-7.59)% and 40.77(13.49-73.86)%, respectively (W = 0, $P < 0.05$). In contrast, no difference in the median (quartiles: 25 and 75%) occurrence of malformed fry was observed at yolk-sac resorption between the 12°C and the 17°C groups, with 7.57(6.44-8.76)% and 5.45(4.48-7.14)%, respectively (W = 11, $P = 0.48$). Similarly, the occurrence of the different types of malformation did not significantly vary among the experimental groups (Fig. S1). At 75 days post-fertilization, fish mean (±SEM) weight tended to be lower in 17°C than in 12°C (0.39 ± 0.02 g *vs.* 0.46 ± 0.05 g), although not significantly ($F_{1,5} = 4.88$, $P = 0.08$).

Offspring behaviour in the novel-tank test

Offspring from thermally stressed mothers displayed weaker emotional responses than controls when individually introduced into a novel-tank (Fig. 2). When considering distance travelled, the temperature X interval interaction tended to be significant ($F_{1,201} = 3.80$, $P = 0.05$, Fig. 2A), and a significant global increase was found between the first 5 min and the last 5 min (interval effect: $F_{1,201} = 4.90$, $P = 0.03$). Distance travelled did not differ

between 12°C and 17°C alevins (non significant temperature effect: $F_{1,5} = 0.002$, $P = 0.96$). Post-hoc tests are detailed in the Fig. 2 legend. The variance associated with the random factor female (4.63) was included in a confidence interval excluding 0, indicating that the random factor was significant. Maximum velocity did not differ between 12°C and 17°C ($F_{1,5} = 0.08$, $P = 0.79$, Fig. 2B). A significant global decrease was observed between the two intervals ($F_{1,201} = 22.89$, $P < 0.001$). No significant interaction was found ($P = 0.56$). The random factor female was significant. When considering angular velocity, the temperature X interval interaction was significant ($F_{1,201} = 5.58$, $P = 0.02$, Fig. 2C). No temperature or interval effects were found. Post-hoc tests are detailed in Fig. 2 legend. No temperature effect, interval effect or significant interaction was found for time spent in thigmotaxis (Fig. 2D). The low variance (0.41) associated with the random factor female indicated a non-significant random effect.

Offspring spatial learning and memory

Offspring from thermally stressed mothers were slower to locate the rewarded arm than controls, when tested in a T-maze (Fig. 3). During the acquisition phase, we did not find any temperature ($\chi^2 = 1.65$, df = 1, $P = 0.20$) or trial effect ($\chi^2 = 12.01$, df = 10, $P = 0.28$) when considering the latency to leave the start-box. The temperature X trial interaction was not significant ($P = 0.15$). The low variances (< 0.001) associated with random factors female and individual indicated non-significant random effects. When considering the latency to make the right choice, we found a significant temperature X trial interaction ($\chi^2 = 19.49$, df = 10, $P = 0.03$, Fig. 3A). The summary of the model indicated a decrease in the latency from the 5th trial compared to trial 1 ($P < 0.05$), in the 12°C group only. These post-hoc effects are detailed in Fig. 3 legend. The fixed factors temperature and trial were not significant (P

= 0.20 and $P = 0.22$, respectively). The random factors female and individual were not significant.

Memory data were considered between trial 11 and three days after. No temperature effect, trial effect or significant interaction was found for the latency to leave the start-box ($P > 0.1$). In 12°C group, the mean (± SEM) elapsed time between opening of the door and fish exit was 164.95 ± 41.48 seconds three days after trial 11 (*vs.* 300.65 ± 92 sec, in trial 11), whereas in 17°C Latency SB reached 407.60 ± 145.15 sec (*vs.* 591.13 ± 152.87 sec in trial 11). The variances associated with random factors female (0.10) and individual (1.58) indicated significant random effects.

The latency to make the right choice tended to decrease between trial 11 and three days later (trial effect: $\chi^2 = 3.25$, df = 1, $P = 0.07$, Fig. 3B). No temperature effect or significant interaction was found. In 12°C group, the mean (± SEM) elapsed time between fish exit from the start-box and entry into the rewarded arm was 296.8 ± 74.57 sec (*vs.* 625 ± 151.14 sec, in trial 11), whereas in 17°C, Latency RC was 607.27 ± 152.16 sec (*vs.* 1009.4 ± 193.62 sec in trial 11). The low variances associated with random factors female (< 0.001) and individual (< 0.01) indicated non-significant random effects.

Three days after the 11th trial, we found a significant temperature X entry choice interaction ($\chi^2 = 5.60$, df = 1, $P = 0.02$, Fig. 3C). A significant choice effect was found ($\chi^2 = 16.02$, df = 1, $P < 0.001$), the elapsed time before entering into the right arm being lower than into the wrong arm. The factor temperature was not significant.

Egg cortisol content

We did not observe any significant difference in the cortisol content of eggs originating from different maternal groups. Median (quartiles: 25 and 75%) cortisol levels did not

differ between 17°C and 12°C unfertilized eggs, with 2.37 (1.84-3.88) ng/g *vs.* 4.11 (1.90-7.13) ng/g, respectively (W = 9, $P = 0.89$).

Gene expression profiling in embryos with different maternal history

Gene expression profiling was performed in eggs and throughout development after maternal exposure to either 12°C or 17°C. The ANOVA resulted in the identification of 47,711 differentially expressed genes throughout development. In contrast, a much lower number of genes were differentially expressed in response to maternal exposure to high temperature (Fig. 4A). Twelve genes exhibited a differential expression in response to temperature while only 5 genes were differentially expressed in response to temperature and among the developmental stages analysed (temperature X stage significant interaction: $P < 0.05$). A total of sixteen genes were thus significantly dysregulated during development in response to maternal exposure to high temperature, one gene (*srsf2a*) being present in both groups. Among these genes, several were of particular interest due to their role in neurodevelopment (*auts2, dpysl5*), neural disorder (*arv1*), and X-linked cognitive disability (*plp2*), as discussed below. Interestingly the expression profiling analysis (Fig. 4B) revealed that the differential expression between groups was especially marked in eggs, and to a lower extend at 5 dpf, while differences were more limited during further development (i.e. hatching and yolk-sac resorption stages). For *auts2* and *dpysl5* maternal mRNA abundance was dramatically lower when females were exposed to high temperature (W = 16, $P < 0.05$; Fig 4C), while *arv1* exhibited an opposite pattern (W = 0, $P < 0.05$). Similarly, *plp2* abundance appeared higher in the 17°C group in eggs and 5 dpf embryos (W = 0, $P < 0.05$; Fig.4C).

Discussion

Our aim was to investigate the effect of a thermal stress, applied to female rainbow trout during the peri-ovulatory period, on offspring behavioural phenotypes. As expected, the thermal stress triggered an increase in embryonic mortality, but not in the occurrence of malformed fry. In addition, fear responses to a novel environment were inhibited in 17°C offspring, which indicates emotional blunting. The thermal maternal stress also impaired spatial learning abilities in progeny. In consistency with these impaired behaviours, we observed a dysregulated expression of embryonic genes involved in neural and cognitive development revealed by a large-scale transcriptomic analysis.

Maternal effects on embryonic survival and development

Our results are in full agreement with previous reports on the deleterious effect of high temperature exposure in peri-ovulatory period on offspring survival in salmonids (rainbow trout: (Aegerter & Jalabert 2004), Atlantic salmon, *Salmo salar*: (King & Pankhurst 2004; King et al. 2003; Taranger & Hansen 1993), Arctic charr, *Salvelinus alpinus*: (Atse et al. 2002)). Despite small sample size (e.g. four females per treatment), differences between treatments were significant at hatching and yolk-sac resorption, but not at eyeing, which is also consistent with the results obtained by Aegerter et al. (2004). In addition, body weight measured at 75 dpf tended to be lower in offspring originating from high temperature-exposed females. This is consistent with previous studies performed on fish, which showed lower offspring survival rates and impaired growth after maternal cortisol administration (Eriksen et al. 2007; Eriksen et al. 2015) or maternal stress exposure (Campbell et al. 1994; McCormick 2009).

354 ## Maternal effects on emotional responses

355 The novel-tank test consisted in observing immediate fish behavioural responses when
356 individually transferred into a novel environment, which is a context known to elicit acute
357 stress responses in various vertebrates including salmonid fish species (Colson et al. 2018;
358 Colson et al. 2015a; Kittilsen et al. 2009; Overli et al. 2005; Rouger et al. 1998; Winberg et
359 al. 2007). Our results show that fishes originating from thermally stressed females were
360 less reactive to the challenging situation than controls. Angular velocity, which represents
361 erratic swimming and is commonly considered as an expression of fish anxiety (Blaser et al.
362 2010; Egan et al. 2009), tended to be lower in 17°C fish during the first 5 minutes of the
363 test. In a previous study performed on wild largemouth bass (*Micropterus salmoides*),
364 mature females were cortisol-injected (Redfern et al. 2017). In line with our results related
365 to lower angular velocity, offspring of treated females exhibited less anxiety, as indicated
366 by decreased thigmotaxis behaviour (e.g. close to the tank walls). The maximum velocity,
367 which is the first escape response commonly observed in isolated fish subjected to the
368 novel-tank test (Champagne et al. 2010; Colson et al. 2015a), was dramatically increased in
369 both groups immediately after the introduction into the novel tank (first 5 minutes). This
370 observed ceiling effect is certainly due to the strength of induced fear, avoiding any
371 possible discrepancy between the two groups for this parameter.
372 While mean distance travelled increased at the end of the test for control fish suggesting a
373 return to normal swimming pattern in this group, 17°C fish exhibited a constant low
374 swimming activity from the start to the end of the test. Existing studies reporting activity
375 levels in prenatally stressed individuals when subsequently subjected to challenging
376 situations are often contradictory. Increased or decreased activity is reported but this

discrepancy can be due to different contexts, intensity or duration of the challenge, species, age, and sex of the individuals tested. Our findings are however consistent with the majority of studies performed in mammals, which showed reduced activity in the offspring of females subjected to different stressors during pregnancy (Fride et al. 1986; Fujioka et al. 2001; Masterpasqua et al. 1976; Patin et al. 2004; Suchecki & Palermo Neto 1991), even though the stress was applied before fertilization in the present work. Interestingly, similar results were also found in fish (Eriksen et al. 2011; Espmark et al. 2008; Sopinka et al. 2014; Tierney et al. 2009). For instance, Sockeye salmon (*Oncorhynchus nerka*) reared from mothers exposed to a chase stressor swam for shorter periods of time (Sopinka et al. 2014). In Atlantic salmon, maternal cortisol exposure increased time spent non-swimming in juveniles (Espmark et al. 2008), and 1.5-year offspring from cortisol-implanted females also exhibited a reduction in the time spent moving compared to the controls during an acute confinement stress (Eriksen et al. 2011). These last studies focussed on the maternal endocrine status at spawning affecting several aspects of progeny behaviour and the results are consistent with the behavioural phenotypes observed here. In fish, thermal stress is known to trigger an increased plasma cortisol level (Quigley & Hinch 2006; Ryan 1995; Zubair et al. 2012). We however did not find any increase in cortisol concentration into the 17°C eggs sampled before fertilization, which is in agreement with observations made by (Sopinka et al. 2014) and (Redfern et al. 2017). This finding rules out the participation of egg cortisol and indicates that the maternal observed effects are triggered by other mechanisms than the direct deposition of cortisol into the egg. In oviparous species, the external embryonic development implies that maternal stress transmission is only possible before fertilization through either egg content in molecules of various nature

(Lubzens et al. 2017), or epigenetic mechanisms. The intergenerational effects reported here are thus more likely due to genomic effects mediated by epigenetics mechanisms and/or specific features of the female gamete including maternally inherited nucleic acids and proteins.

In mammals, there is growing evidence that stress during pregnancy causes attention deficits and depressive disorders (Ronald et al. 2010; Talge et al. 2007), as well as impaired emotional behaviours of adult offspring (Fride et al. 1986; Shiota & Kayamura 1989; Vallée et al. 1999; Zagron & Weinstock 2006). The lack of behavioural reaction to the challenge observed in 17°C fish suggests a global emotional blunting and an attention deficit, resembling the depressive-like symptoms described in prenatally stressed rodents (Morley-Fletcher et al. 2003; Poltyrev et al. 2005). In these studies, animals do not further respond to stressful stimuli, decrease explorative behaviour and their activity implying a form of resignation to an adverse uncontrollable situation. In a previous experiment, we noticed the absence of fear from a novel object (e.g. neophobia) in offspring from thermally stressed females (V. Colson, unpublished data). The absence of neophobia was likewise observed in suffering rainbow trout after being exposed to a nociceptive stimulus (Sneddon et al. 2003) and can be interpreted as a lack of attention for the environment. In the present study, the weaker emotional responses, as indicated by a decrease in angular velocity upon initial exposure to the novel tank and an absence of resumed ambulation at the end of the test might be explained by attention alterations due to maternal stress. Fish originating from thermally stressed mothers may be predicted to display a reduced ability to cope with their environment, since emotion and attention deficits might be major disadvantages in adverse or changing environments (Bijlsma & Loeschcke 2005). In

rainbow trout, first feeding is a key-stage during which fear-related behaviour, such as fast-start swimming, 'freezing', hiding and exploring are essential traits for fry survival. Therefore, hypo-active behaviour, as shown in the present study, could have direct impacts on fish survival chances under natural conditions.

Maternal effects on cognition

In the present experiment, we observed cognitive disorders in 17°C fish. Fry from mothers exposed to suboptimal temperature during late oogenesis were slower to locate the rewarded area in the spatial learning task. This finding is consistent with studies performed in other oviparous species (birds: (Guibert et al. 2013; Lindqvist et al. 2007) and fish: (Eaton et al. 2015; Roche et al. 2012)), showing cognitive disorders in offspring of mothers stressed before fertilization compared to offspring of non-stressed animals. In three-spined sticklebacks, offspring of predator-exposed mothers located the food reward more slowly than offspring of unexposed mothers (Roche et al. 2012). Female guppies (*Poecilia reticulate*) exposed to routine husbandry procedures that induced only a minimal elevation of cortisol, produced offspring that failed to associate a colour cue and food reward (Eaton et al. 2015). Conversely, in brook trout *Salvelinus fontinalis*, maternal cortisol consumption and handling did not impact spatial learning or memory in 6 month-old offspring (Cortez Ghio et al. 2016). This inconsistency might indicate that maternal effects on fish cognition are context-dependent or different depending on the type of stress used. Except for the above examples, very few studies have investigated intergenerational effects on fish cognition, and to our knowledge our findings are the first to show that a thermal maternal stress is linked to emotional and cognitive disorders.

When the learnt association was recalled 3 days after the last trial, 12°C fish were quicker to reach the right arm than 17°C fish, while the wrong arm was reached in both groups after a longer latency. Interestingly, the duration needed to reach the correct arm tended to be shortened during the memory test comparing to the latency measured at the last training trial within the two groups. This indicates that impaired cognition due to the thermal maternal stress concerned only the acquisition phase but not the retention pathway. Indeed, although 12°C fish were quicker to obtain the reward, fish from both groups remembered the correct location. During the 2-day break between the last trial and the memory test, fish were not fed as well as between each trial, the food being obtained only as a reward during the entire experimental procedure. Thus a high feeding motivation was observed at the moment of the recall, as also indicated by the short latency to leave the start-box, although the effects were not significant. Therefore, when highly motivated, rainbow trout demonstrated a memory capacity of at least 3 days in this spatial learning paradigm, which is consistent with a previous experiment showing that this fish species likewise remembered for 3 days an association by appetitive classical conditioning (Nordgreen et al. 2010).

In summary, fish originating from thermally stressed mothers were slower than controls in the spatial learning task. Although they were able to remember the food location after a 2-day break, they seemed to be less motivated, as indicated by the longer latency to reach the goal, which is consistent with the global blunting emotional responses observed in the novel-tank test in 17°C group. Cognitive abilities are critical for aquaculture fish since they need to anticipate specific events (e.g. food delivery) in order to reduce stress triggered by an unpredictable environment (Jones et al. 2012). Moreover, the ability to habituate to

repeated and fearful, but harmless, stimuli (e.g. repeated fishing linked to aquaculture practices) (Lieberman 2000) can be extremely useful for fish in order to avoid chronic stress under aquaculture conditions. It is thus highly beneficial for cultured fish to enhance or at least to preserve learning abilities (e.g. conditioning and habituation). Adaptive capacities remain a key component of fish welfare under breeding condition and this study reveals detrimental effects of heat maternal exposure on these capacities in the offspring.

Maternal effects on embryonic gene expression

Our results on the impact of thermal stress during the peri-ovulatory period (i.e. before fertilization) on offspring behaviour are similar to results obtained in mammals during pregnancy (Szuran et al. 1994; Talge et al. 2007; Vallee et al. 1999; Weinstock 2005; Zagron & Weinstock 2006). In humans, studies have shown that if a mother is stressed while pregnant, her child is at increased risk of having a range of problems, including emotional problems, attention deficits, and impaired cognitive development. These behavioural patterns are very similar to those observed in the present experiment. There is growing evidence for non-genetic effects of maternal experience on offspring in rodents (Weiss et al. 2011), and more recently in fish (Mommer & Bell 2014). Here, we used a robust methodology (i.e. microarray) and a conservative statistical approach to reveal the most relevant molecular players despite the low number of females that we simultaneously ovulated in both experimental groups. Among the sixteen differentially expressed genes, four genes are known to participate in neurodevelopment (*auts2, dpysl5*) or associated with neural/cerebral disorders (*arv1*) and X-linked cognitive disability (*plp2*). In humans, *AUTS2* is officially named *activator of transcription and developmental regulator* according to the official gene nomenclature (HGNC:14262 https://www.genenames.org/). It was

previously named *autism susceptibility candidate 2* (Sultana et al. 2002). The human *AUTS2* locus is associated with a wide diversity of neurological disorders, indicating that AUTS2 is involved in neurodevelopment (see (Hori & Hoshino 2017a) for review). Several forms (splice variants) of the genes are expressed in the mouse during development, including during *in utero* development (Gao et al. 2014; Hori et al. 2014). In zebrafish, *auts2* is also embryonically expressed and found in the forebrain, midbrain and hindbrain at 24 hours post-fertilization (Oksenberg et al. 2013). This early embryonic pattern in zebrafish and mouse is consistent with the embryonic expression profile reported here throughout rainbow trout development. Interestingly, *Auts2* expression in the mouse brain is especially high in regions associated with higher cognitive functions, including in the prenatal brain (Bedogni et al. 2010). Functional analyses conducted in zebrafish (*Danio rerio*) confirmed the major role played by *auts2* in fish neurodevelopment (Oksenberg et al. 2013). Knock down of *auts2* in zebrafish resulted in considerably less developing neurons in the optic tectum, retina, and cerebellum. Interestingly, observed phenotypes were less severe when the morpholino (MO) used was directed against a splice junction rather than the translation initiation site, indicating that maternally-inherited *auts2* mRNA played an important role in Auts2-mediated neurodevelopment. Together, these observations are fully consistent with our data, especially the profiles of *auts2* maternal RNA shown in Fig. 4B and suggest that the intergenerational effects of maternal exposure to high temperature could be mediated, at least in part, by differences in egg content in *auts2* messenger RNA. Data in mouse and zebrafish indicate that *Auts2* acts as a transcriptional regulator for neural development through interactions with several genes related to brain development and neurological disorders. More specifically, Auts2 appears to be participating in neuronal

514 migration and neurite extension and is critical for the acquisition of neurocognitive
515 function (see (Hori & Hoshino 2017b) for review). Behavioural phenotypes observed in
516 Auts2 heterozygous mutant mice are characterized by lower anxiety-like behaviour and
517 impaired memory (Gao et al. 2014; Hori et al. 2015). These phenotypes are strikingly
518 similar to the phenotypes observed here after maternal exposure to high temperature and
519 characterized by emotional numbing (i.e. lower angular velocity and absence of locomotor
520 activity modifications under stressful situation) and impaired learning abilities (i.e. slower
521 to locate a food-reward than controls in a T-maze). In addition to *auts2*, we also observed
522 the dysregulation of *dpysl5, (dihydropyrimidinase-like 5*) a member of CRMP (collapsing
523 response mediator protein) family thought to be involved in neural development (Veyrac et
524 al. 2011). Together these observations strongly suggest that the dysregulation of
525 neurodevelopment genes expression, especially *auts2,* but also *dpysl5*, in eggs and embryos
526 participate in mediating the intergenerational effects on offspring behaviour observed here
527 after exposing rainbow trout females to high temperature.
528 The transcriptomic analysis also revealed the differential expression, in response to
529 maternal exposure to high temperature, of genes associated with neural/cerebral disorders
530 (*arv1*) and X-linked cognitive disability (*plp2*). In an attempt to better understand genes
531 affecting human brain function, a recent whole-exome sequencing study in 143 families
532 resulted in the identification of 68 recessive genes associated with neurological disorders
533 (Alazami et al. 2015). Among those genes was *ARV1*, which was also associated with
534 autosomal recessive epileptic encelopathy in another study (Palmer et al. 2016). We also
535 observed a dysregulation of *plp2* in response to maternal exposure to high temperature. In
536 humans a polymorphism in *PLP2* (*Proteolipid protein 2*) promoter was associated with X-

linked Mental Retardation (XLMR) (Zhang et al. 2007). While the roles of *arv1* and *plp2* in fish are currently unknown, the identity and suspected roles of these genes in humans are consistent with the differential abundance of the gene and the emotional blunting and impaired learning abilities observed in the present study.

The genome-wide transciptome analysis also revealed the dysregulation of several other genes, including a so far uncharacterized gene (*cxxcl1l*) that exhibits a strong differential expression in eggs from different maternal origin. These genes are likely to mediate, or at least to participate, in the intergenerational effect of maternal exposure to high temperature observed here. Further analyses are needed to decipher the specific contribution of these genes to the phenotypes reported here.

Together, our results revealed the dysregulation of several genes that are important for the development of cognitive abilities in response to maternal exposure to high temperature. The identity of these genes is consistent with the behavioural phenotypes observed in fry originating from thermally stressed mothers. Additional studies aiming at characterizing possible epigenetic modifications, gene expression and neurotransmitters activity in target brain structures are still needed to further understand the mechanisms mediating the observed behavioural modifications subsequent to thermal maternal stress in rainbow trout.

Conclusions

Here we show that fish originating from thermally stressed mothers exhibit emotional and cognitive disorders, which would be a major disadvantage under suboptimal or fluctuating environments. These impaired behavioural phenotypes are associated with the

559 dysregulation of several genes known to play a major role in neurodevelopment. This is
560 especially true for *auts2*, a key gene for neurodevelopment, more specifically neuronal
561 migration and neurite extension, and critical for the acquisition of neurocognitive function
562 in fish and mammals. In addition to *auts2*, our analysis revealed the dysregulation of
563 another neurodevelopment gene (*dpysl5)* as well as genes associated with cognitive
564 disorders in humans (*arv1*, *plp2*). Our study also revealed that some of the observed
565 intergenerational effects are associated with a major dysregulation of several maternally-
566 inherited mRNAs accumulated into the egg. Together, our observations shed new light on
567 the intergenerational determinism of fish behaviour and associated underlying
568 mechanisms. Our results address an important question for wild or cultured fish adaptive
569 capacities in the context of climate warming.

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Figure 1(on next page)

Embryonic mortality

Effects of rearing temperature before ovulation (12°C and 17°C) on the occurrence of embryonic moratlity (%) at different developmental stages (eyeing, hatching and yolk-sac resorption). Values are medians (quartiles: 25 and 75%). * $P < 0.05$: significant difference between treatments (n= 4).

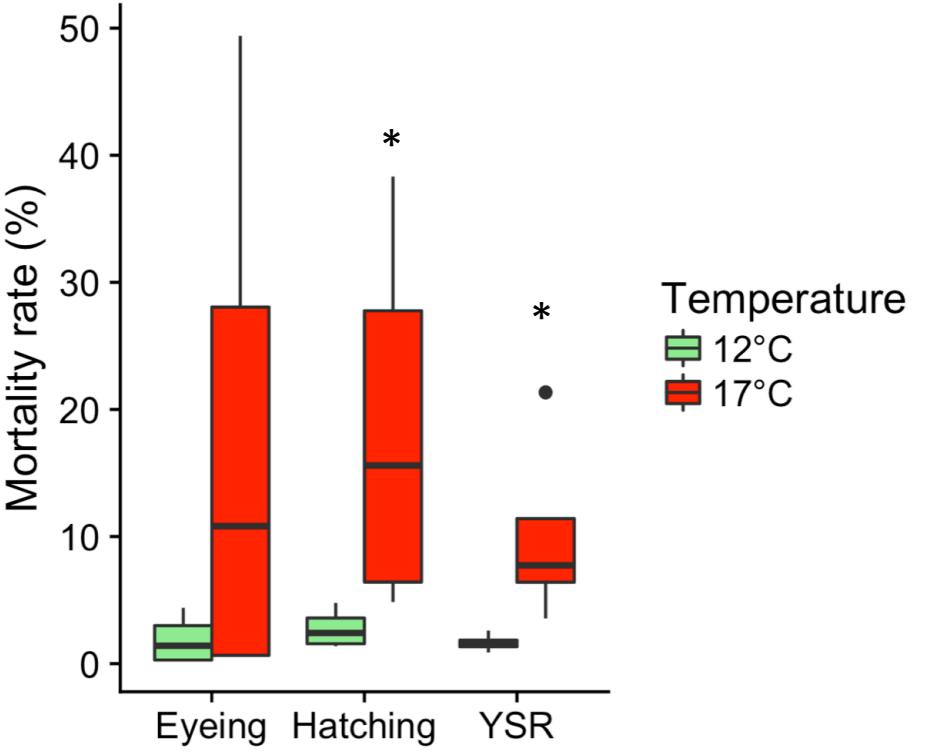

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Figure 2(on next page)

Swimming behaviour in novel-tank test

Swimming behaviour of 75-76 dpf progeny from mothers exposed to 12°C and 17°C before ovulation, video-filmed for 30 min in social isolation in a novel environment. Behaviours were recorded during the first 5-min interval of the test (5 min) and the last 5-min interval of the test (30 min). (A) Total distance travelled (cm). (B) Maximum velocity (cm/sec). (C) Angular velocity (°/sec). (D) Time spent in the border over the 5 minutes (% of time). Values are means and their associated mean standard error (SEM) (n=15). Significant main effects and interactions are indicated (NS: non significant, * $P< 0.05$, *** $P< 0.001$). Random female effect is indicated (* $P< 0.05$). Different letters indicate significant differences shown by post-hoc tests ($P< 0.05$) or a tendency (t $0.05 < P< 0.1$).

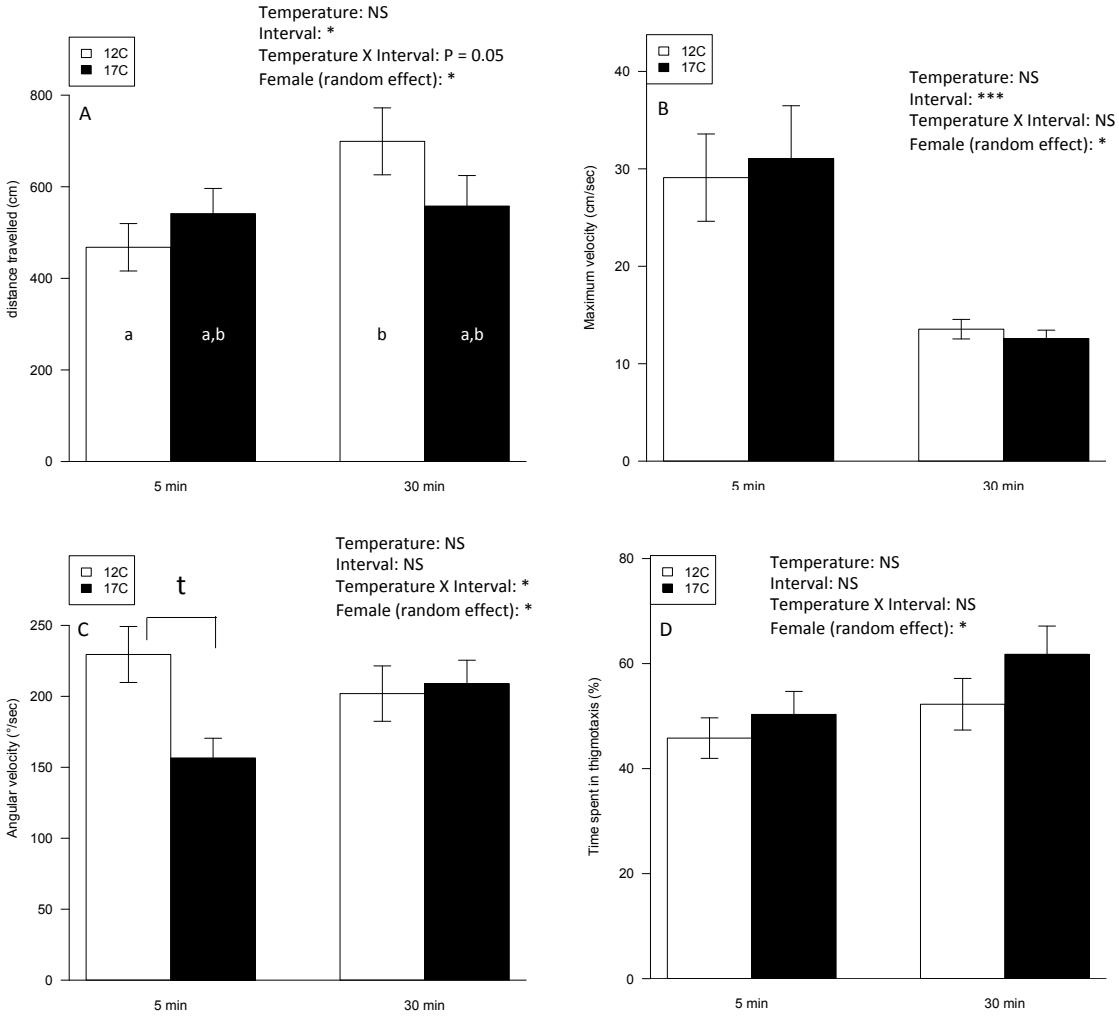
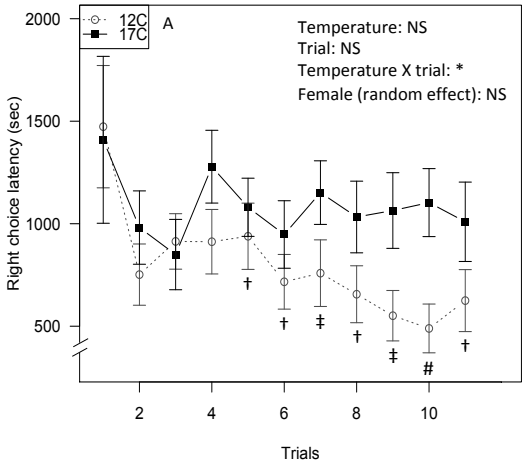
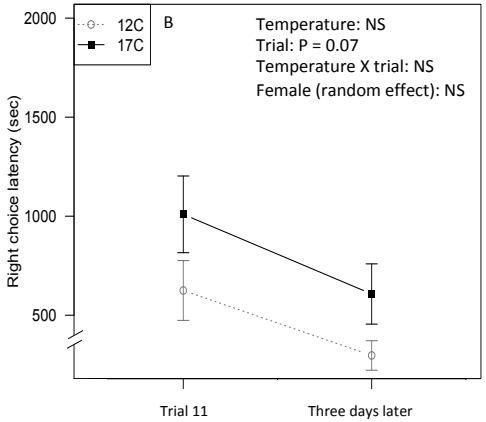


Figure 3(on next page)

Spatial learning and memory

Latency (seconds) to make the right choice by reaching the rewarded arm of a T-maze in progeny from mothers exposed to 12°C and 17°C before ovulation. Latencies were measured (A) during the acquisition phase within 11 successive trials, lasting 1800 seconds each, (B) three days after trial 11, to measure fish memory, and (C) before fish entry into either the right or the wrong arm, three days after the 11thtrial. Values are means and their associated mean standard error (SEM) (n= 5). Main effects and significant interactions are indicated (NS: non significant, * $P< 0.05$). Non-significant random female effect is indicated. † $P< 0.05$, ‡ $P< 0.01$, # $P< 0.001$: significant differences from Trial 1, within 12°C. Different letters indicate significant differences between 12°C and 17°C shown by post-hoc tests ($P< 0.05$).





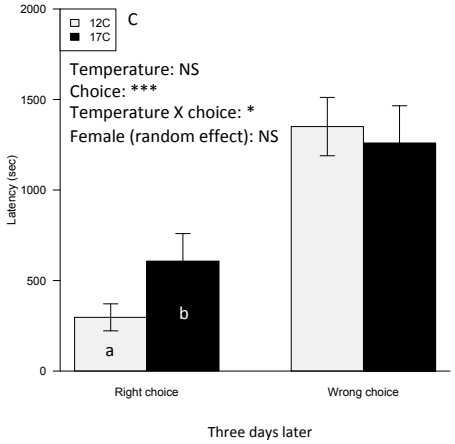


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Figure 4(on next page)

Microarray analysis of gene expression in eggs and progeny originating from mothers exposed to either 12°C or 17°C during the peri-ovulatory period.

A.Venn diagram representing the number of differentially expressed genes. Two-way-ANOVA performed using maternal temperature and developmental stage as fixed factors. Benjamini-Hochberg corrected p values < 0.05. Gene symbols are shown when a significant effect was obtained for Temperature and Temperature X Stage interaction. All corresponding data are presented in supplementary data file 1.**B.**Supervised clustering analysis of the expression profiles of the 12 genes significantly dysregulated due to the temperature effect (panel A). Data were median-centered and an average linkage clustering was performed. Neurodevelopment genes and genes related to human cognitive disorders are shown in purple. **C.**Boxplot representation of gene expression profiles of neurodevelopment genes (*auts2*and *dpyls5a*) and genes related to human cognitive disorders (*arv1*and *plp2*) corresponding to the data delineated in purple on panel B. AU: arbitrary units.