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Stickleback embryos use ATP-binding cassette transporters as a buffer against exposure to maternally derived cortisol

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Offspring from females that experience stressful conditions during reproduction often exhibit altered phenotypes and many of these effects are thought to arise owing to increased exposure to maternal glucocorticoids. While embryos of placental vertebrates are known to regulate exposure to maternal glucocorticoids via placental steroid metabolism, much less is known about how and whether egg-laying vertebrates can control their steroid environment during embryonic development. We tested the hypothesis that threespine stickleback (*Gasterosteus aculeatus*) embryos can regulate exposure to maternal steroids via active efflux of maternal steroids from the egg. Embryos rapidly (within 72 h) cleared intact steroids, but blocking ATP-binding cassette (ABC) transporters inhibited cortisol clearance. Remarkably, this efflux of cortisol was sufficient to prevent a transcriptional response of embryos to exogenous cortisol. Taken together, these findings suggest that, much like their placental counterparts, developing fish embryos can actively regulate their exposure to maternal cortisol. These findings highlight the fact that even in egg-laying vertebrates, the realized exposure to maternal steroids is mediated by both maternal and embryonic processes and this has important implications for understanding how maternal stress influences offspring development.

1. Introduction

The physiological status of females during reproduction can produce long-lasting effects on her offspring. For example, the exposure of females to adverse conditions during pregnancy is associated with phenotypic effects on her offspring such as altered growth, physiology and behaviour [1,2]. These maternal effects are frequently referred to as 'developmental programming' [3], with some effects being potentially adaptive [4,5], whereas others are likely maladaptive [6]. Mechanistically, many of the effects of maternal stress on offspring are thought to arise as a result of increased exposure of offspring to maternally derived glucocorticoids [5]. Thus, to fully understand how maternal stress influences offspring development, we need to understand how embryonic exposure to maternal glucocorticoids is regulated.

In placental mammals, the placenta modulates foetal exposure to maternal glucocorticoids by metabolizing glucocorticoids as they pass from maternal circulation into foetal circulation. The enzyme primarily responsible for this metabolism is 11β-hydroxysteroid dehydrogenase type II (11β HSD), as it catalyses the inactivation of glucocorticoids [7] and results in an 80–90% metabolism of glucocorticoids as they cross the placenta [8,9]. This metabolic buffer plays a vital role in modulating the effects of maternal stress as evidenced by the number of effects that arise when this buffer is inhibited pharmacologically [10], knocked out genetically [11] or overwhelmed with synthetic glucocorticoids [12]. The idea that maternal glucocorticoids do not always reach developing offspring without first being metabolized suggests that some of the effects of maternal stress on offspring development might occur without direct

glucocorticoid exposure. Research aimed at untangling the direct effects of glucocorticoid exposure from the indirect effects that arise owing to glucocorticoid-mediated changes in maternal physiology following stress demonstrate that many of the effects on offspring do not require direct glucocorticoid exposure [11]. Stress-induced changes in maternal physiology, such as reduced sex steroid production and altered lipid metabolism, may elicit effects on offspring development even if glucocorticoids themselves do not reach the embryo as an active form. Ultimately, the effects of maternal stress on offspring are likely a combination of direct glucocorticoid effects and indirect effects mediated by changes in maternal physiology. This work on how maternal glucocorticoids influence offspring development highlights the vital role embryonic processes play in modulating the effects of maternal steroids.

Until recently, it was generally thought that the embryos of egg-laying vertebrates had minimal ability to regulate exposure to maternally derived steroids that can accumulate in lipid-rich eggs [13], but studies are now demonstrating that these embryos also use metabolism to regulate their exposure to maternal steroids [14]. Most of what we know about how vertebrate embryos regulate their exposure to maternal steroids implicates extraembryonic membranes, including the placenta, as the most important tissues in this process [15,16]. However, embryos of non-amniotic vertebrates (fish and amphibians), which lack these extraembryonic membranes [17], are also exposed to maternally derived steroids (progesterone [18]; oestradiol [19]; testosterone [20]; cortisol [21–27]) and are sensitive to steroids [28,29]. Moreover, despite lacking extraembryonic membranes, steroids decline during development in fishes [18,30], which sets up the possibility that fish embryos may be capable of modulating their exposure to maternal steroids, even though they do not have extraembryonic membranes.

In addition to metabolism, other embryonic processes can be involved in regulating exposure to maternal steroids. For example, mammals use specialized transport proteins to actively transport steroids and their conjugates [31]. ATP-binding cassette (ABC) transporters and solute carrier proteins can use steroids and metabolites as substrates, thus the expression of transporters can impact movement of these compounds within the body [32]. Many of these transporters are present within the placenta and their substrate specificity and cellular distribution play a role in regulating which steroids and conjugates are moved from maternal circulation, through the placenta, and into foetal circulation (and vice versa) [31]. In fish, ABC transporters are considered the 'first line of defence' against uptake of xenobiotics from the aquatic environment [33]. This arises from that fact that ABC transporters are capable of transporting a wide variety of xenobiotics and modulating toxicity by preventing exposure [34]. Recently, ABC transporters have also been shown to play an important role during embryonic development as they protect developing fish embryos from a variety of exogenous chemicals present in the water [35]. Taken together, the metabolism of maternal steroids and the transport of steroids/metabolites are likely to influence the exposure of developing embryos to maternal steroids and subsequently influence how maternal steroids impact development. Both of these processes have been suggested to be vital, protecting developing embryos from the potentially detrimental effects of increased glucocorticoid exposure during maternal stress [36]. Here, we test the hypothesis that embryos of non-amniotic vertebrates can modulate their exposure to maternal glucocorticoids and buffer themselves from the effects of maternal stress using threespine stickleback (*Gasterosteus aculeatus*) as a model. We examined the fate of maternally derived cortisol by treating unfertilized eggs with tritiated cortisol and subsequently: (i) characterized the movement of cortisol during early development, (ii) tested for the presence of cortisol metabolites, and (iii) examined the effect of inhibiting ABC transporters on the movement of cortisol. Additionally, we used RNAseq to test whether the embryonic transcriptional response to increased levels of cortisol was similar to the transcriptional response to maternal stress in sticklebacks [37].

2. Methods

(a) Animal collection and care

Adult sticklebacks were collected from Putah Creek, CA during the spring of 2013 and 2014. Fish were transported to the University of Illinois, where they were housed in 37 l tanks in groups of five to eight. Fish were held on a natural photoperiod at 20°C and fed frozen bloodworms, Mysis shrimp, cyclopeeze and brine shrimp ad libitum. Once males exhibited reproductive morphology (red throat colour), they were moved to separate 9 l tanks. Females were monitored for gravidity, and eggs were collected by gently squeezing the abdomen.

(b) Study I—movement and metabolism of cortisol

First, we verified that cortisol was taken up into the unfertilized eggs following immersion in a cortisol-containing solution, as this is a common technique used to administer steroids to fish eggs [38]. By validating an uptake of tritiated cortisol, subsequent studies could use tritiated cortisol to trace the movement and metabolism of cortisol during development. Ten clutches were immersed in 50 ml of water (10% solution of Instant Ocean™) containing 20 000 cpm of tritiated cortisol (specific activity = 95.4 Ci mmol^{-1}) (Perkin-Elmer, Boston, MA) per 100 µl, which is equivalent to 72 pg cortisol per 100 µl. To avoid solvent effects, the ^{3}H-cortisol that was dissolved in ethanol was added to a glass beaker, dried under a stream of air, reconstituted in water and counted to verify concentration. Each clutch developed in 50 ml of this solution, which contained a total of 10 000 000 cpm of cortisol. Subsets of 10 eggs per clutch were collected after 5, 10, 20, 30 min of immersion. Eggs were rinsed three times and homogenized. Radioactivity was extracted from this homogenate by adding 200 µl of 100% methanol and vortexing for 30 s. The samples were then placed at −20°C for 2 h and centrifuged at 2000 rpm for 10 min. The supernatant was added to 3 ml of scintillation fluid and counted on a Beckman 6500 scintillation counter.

Upon verifying that cortisol was taken up following immersion, we examined the movement and metabolism of this cortisol following fertilization. Here, unfertilized eggs were immersed in water containing 20 000 cpm of cortisol per 100 µl and then fertilized. Testes were removed from a euthanized male (MS-222 overdose), macerated in two drops of water and sperm applied to eggs for 3 min. Following fertilization and three rinses with fresh water, eggs were transferred to 50 ml of water still containing 20 000 cpm of cortisol per 100 µl. Keeping eggs in a solution that still contained cortisol allowed us to test whether embryos were able to regulate cortisol levels against a concentration gradient as this would provide a stronger test of the hypothesis that embryos were actively modulating cortisol levels. A subset of 10 eggs was collected at this point to verify cortisol uptake, whereas the remaining eggs were allowed to develop for 72 h, because maternal stress has been shown to influence embryonic gene transcription over this developmental period [37]. After

72 h, eggs were collected and stored at −20°C until radioactivity levels were quantified, and metabolism was characterized via thin layer chromatography (TLC). All steroid standards were purchased from Steraloids Inc. (Newport, RI).

Levels of radioactivity were quantified using the methanol extraction described above. Owing to low levels of radioactivity within eggs after 72 h of development, TLC was conducted on the water in which the eggs developed to test for the movement of metabolites out of the egg and into the water. Steroids were extracted from the water using solid phase extraction with C18 Sep-pak cartridges (Waters Inc., Milford, MA) that had been primed with 5 ml methanol followed by 5 ml distilled water [39]. After the sample had dripped through at a rate of approximately 2 ml min^{-1}, the cartridge was washed with 5 ml of distilled water, steroids were eluted with 5 ml of diethyl ether and steroid conjugates were eluted with 5 ml of 100% methanol. Eluates were dried under nitrogen gas and suspended in 100 µl of 100% methanol. A 5 µl aliquot was counted to quantify radioactivity levels. Only the ether fraction (free steroids) contained radioactivity, so this fraction was used in TLC ($n = 3$). The remaining 95 µl were spotted onto a 4 × 8 cm, aluminium-backed TLC plate (Sigma 70 643) 1 cm from the bottom of the plate. Adjacent to the sample, standards of cortisone, 11 deoxycortisol, 20β dihydrocortisone [40] and cortisol were spotted onto the plates to serve as markers for the location of radioactive metabolites. Plates were developed in chloroform : methanol (95 : 5) [41] until the solvent front reached the top of the plate and then were air-dried before being partitioned into sections and counted. A 1 × 8 cm lane that contained the entire developed sample was cut from the plate. This lane was then cut into 16 0.5 cm × 1 cm sections. The top 15 sections were individually placed into 3 ml scintillation fluid, and radioactivity levels counted to compare the movement of radioactivity with known standards [42].

(c) Study II—transcriptional response to exogenous cortisol

The transcriptional response of embryos to exogenous cortisol was investigated to test the hypothesis that the effects of maternal stress on embryonic gene expression [37] were mediated by increased cortisol exposure. This experimental approach largely mirrors a similar experiment that demonstrated that maternal stress (exposure to a predator) can alter embryonic transcriptional profiles 72 h post-fertilization [37]. Six clutches of eggs were each equally divided into three treatment groups (control, low cortisol, high cortisol). Eggs were treated by immersion for 30 min in water that contained 0, 500 or 1000 pg per 100 µl, respectively. These treatments were estimated to raise cortisol levels to 11, 15 or 19 ng g^{-1}, which represent baseline, predator induced and super physiological levels of maternally derived cortisol, respectively [30,43,44]. Following immersion, eggs were fertilized (one male per clutch) and allowed to develop in 50 ml of water for 72 h.

To isolate enough RNA for RNAseq, 10 embryos from each clutch/treatment were pooled prior to RNA extraction for a total of 18 pools (six clutches × three treatments). Total RNA was extracted from each pool using Trizol® according to the manufacturer instructions and quality analysed on an Aligent Bioanalyser (Aligent Technologies, Palo Alto, CA). Samples were submitted to the Roy J. Carver Biotechnology Center at the University of Illinois for sequencing on an Illumina HiSeq 2000 (TruSeq SBS sequencing kit).

RNAseq data processing—FastQC was used to assess read quality and FastX toolkit was used to filter low quality reads and residual adaptor sequences (hannonlab.cshl.edu/fastx_toolkit/). RNAseq produced an average of 20 million reads per sample. We aligned reads to *G. aculeatus* reference genome (the repeat masked reference genome, Ensembl release 73), using TopHat (2.0.8) and Bowtie (2.1.0). Reads were assigned to features according to the Ensembl release 73 gene annotation file (ftp://ftp.ensembl.org/pub/release-73/gtf/gasterosteus_aculeatus/). For TopHat alignments, we designated library type 'first strand' and otherwise used default parameters.

Identifying differential expression—to estimate differential gene expression we used both Tuxedo [45] and count-based differential expression analysis protocols. Both protocols require each sample's (control (6), low-dose (6), high-dose (6)) reads to align to the reference genome.

In the Tuxedo protocol, cufflinks (2.2.0) reconstructed the transcriptome assembly of each sample by assembling reads into transcripts and estimating their abundance. Later cuffmerge was used to merge all cufflinks assemblies into a single high-quality assembly containing both novel and known isoforms. Finally, cuffdiff [45] was used to call differential gene expression using each treatment group (control, low-dose, high-dose) samples alignments and merged transcriptome assembly.

In the count-based protocol, HTSeq was used to calculate reads counts per gene. Later, we assessed differential expression between treatments groups (control, low-dose, high-dose) using R software package edgeR. This approach uses negative binomial distribution to model counts distribution per gene across sample. Count data were normalized by library size and library composition (using 'calcNormFactors' function). A gene was included in the differential expression analysis if the number of counts was greater than or equal to two count per million in any sample. We computed a common dispersion estimate followed by tagwise (genewise) dispersion estimation. Finally, to call differential expression between treatment groups, both 'exactTest' and 'glm' approaches were used.

(d) Study III—ATP-binding cassette transporters as a mediator of cortisol movement

A final study was conducted to test whether inhibiting ABC transporters affected the movement of cortisol during the first 72 h of development. For this study, seven clutches of eggs were divided into two treatments. Half of the eggs were fertilized and allowed to develop in water containing 20 000 cpm of tritiated cortisol. The other half of the eggs were fertilized (by the same male as their siblings) and allowed to develop in water containing 20 000 cpm of cortisol *PLUS* 10 µM cyclosporin A (general inhibitor of ABC transporters in fish embryos) [35]. We selected this dose of cyclosporin A based on a study in zebrafish embryos which showed that cyclosporin A produced effects similar to morpholino knock-down of *abcb4*, which was identified as the primary regulator of xenobiotic uptake in embryos [35]. While cyclosporin A is not as specific to a particular ABC transporter as some other available inhibitors, this potential ability to block several transporters was viewed as a benefit for our initial attempts to demonstrate that the efflux of steroids from eggs was indeed an active process and not diffusion. Five eggs from each clutch/treatment were collected after 6, 24, 48 and 72 h of development. Radioactivity levels within these pools of five eggs were quantified via methanol extraction.

(e) Statistical analyses

To examine if levels of radioactivity changed within eggs during development (eggs sampled after 10 min versus eggs sampled after 72 h), a mixed model ANOVA was used. Sampling point was included as a fixed factor, whereas clutch of origin was included as a random variable. A similar analysis was used to investigate the effect of cyclosporin A on cortisol levels, but treatment (control versus inhibitor) was added as a fixed effect. These analyses were conducted in SAS v. 9.3 (SAS Institute, Cary, NC).

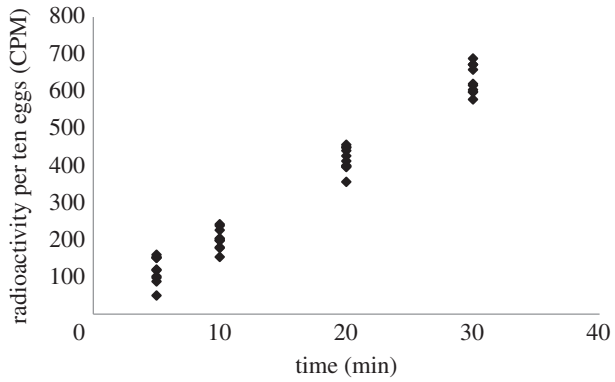

Figure 1. Time-dependent uptake of 3H-cortisol into unfertilized stickleback eggs.

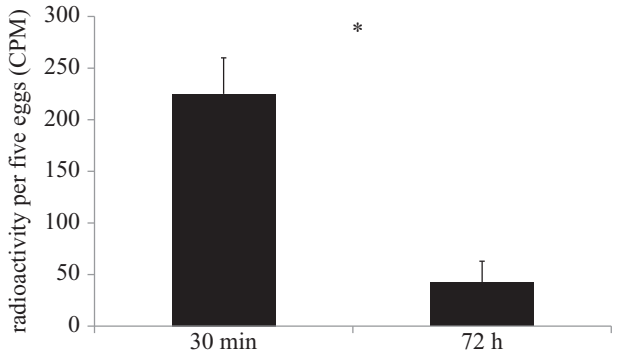

Figure 2. ^{3}H-cortisol levels in stickleback eggs developing immersed in a solution containing 20 000 CPM cortisol per 100 µl. Asterisk designates a significant difference of $p < 0.05$.

3. Results

(a) Cortisol levels in *Gasterosteus aculeatus* eggs immersed in cortisol initially rise but then drop

When eggs were immersed in a solution containing ^{3}H-cortisol, cortisol levels rapidly increased (figure 1), which suggests that steroids can readily move into eggs, and that the lipid-rich yolk can act as a sink [46]. However, by 72 h, ^{3}H-cortisol within eggs dropped significantly, even when eggs were continuously immersed in ^{3}H-cortisol ($F_{1,5} = 119.13$, $p < 0.0001$, figure 2).

(b) Exogenous cortisol is not metabolized by *Gasterosteus aculeatus* embryos

One possible explanation for this decline is that cortisol was metabolized into a form that moved out of the egg. In that case we should be able to detect metabolites in the incubation water surrounding the eggs. However, the TLC analysis provided no evidence that metabolites of cortisol were present in the incubation water, as essentially all detectable radioactivity migrated with the cortisol standard (figure 3).

(c) Inhibition of ATP-binding cassette transporters reduces efflux of cortisol

Another mechanism by which cortisol could move out of the egg is via active transport. To test this hypothesis, we applied an ABC transporter inhibitor (cyclosporin A). Inhibition of

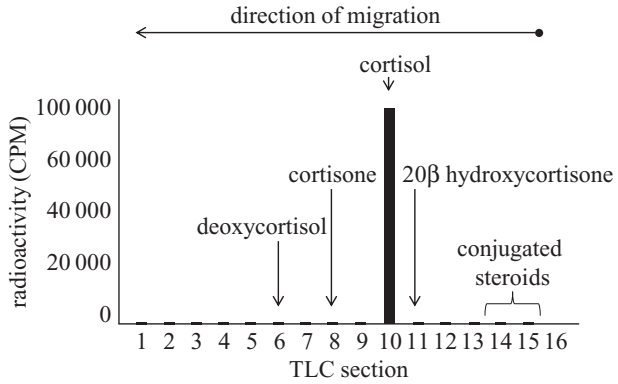

Figure 3. TLC separation of free steroids present in the incubation water following 72 h of development. Sections that contain internal standards are marked with an arrow. Only the section containing the cortisol standard contained radioactivity.

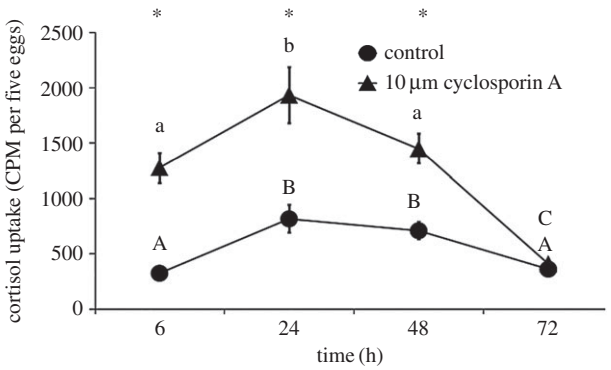

Figure 4. Effect of ABC transporter inhibition on cortisol uptake in developing *G. aculeatus* embryos during the first 72 h following fertilization. Eggs were developed for 72 h in a solution containing 20 000 cpm cortisol per 100 µl. Lower case letters designate significant differences in the inhibited group, whereas upper case letters designate significant differences in the control group. Asterisks designate significant differences between the inhibited and control groups for a give sampling point. *$p < 0.05$.

ABC transporters by cyclosporin A resulted in elevated levels of cortisol within the egg early in development, but levels dropped to levels comparable to the control group by 72 h of development (figure 4). There were significant effects of sample period ($F_{3,42} = 78.34$, $p < 0.0001$) and presence of the inhibitor ($F_{1,42} = 224.91$, $p < 0.0001$), as well as an interaction between the sample period and inhibitor presence ($F_{3,42} = 24.12$, $p < 0.0001$) on levels of cortisol within the egg.

(d) Exogenous cortisol does not influence transcriptional profiles of embryos

The RNASeq data suggest that 17 251 genes out of the possible 22 456 genes in the stickleback genome were expressed in 72-h embryos. However, we did not detect any differences in gene expression between embryos that were treated with cortisol compared with the control group. In other words, immersing eggs in cortisol did not affect the expression of any of the 17 251 genes that were present in 72-h embryos because transcription levels in the control group were the same as in the treatment groups. Quality assessments confirmed that the failure to detect differentially expressed

genes was not the result of technical problems (see electronic supplementary material).

4. Discussion

Results from these studies provide evidence that developing fish embryos can modulate their exposure to maternally derived cortisol. Our findings are consistent with previous studies that have shown that fish eggs take up steroids from their incubation environment [38] and that the steroid content of eggs drops soon after fertilization [18,30]. By tracing tritiated cortisol following fertilization, we demonstrated that *G. aculeatus* embryos develop the capacity to transport steroids out of the eggs soon after fertilization as evidenced by the drop in radioactivity levels within embryos despite the fact that they remained immersed in solution with cortisol. Our examination of whether cortisol might be metabolized prior to being transported from the embryo found no evidence for cortisol metabolites. Instead, our results suggest that stickleback embryos can clear cortisol via ABC transporters: when ABC transporters were inhibited, the accumulation of cortisol within the egg increased. Remarkably, embryos from both the control and inhibited groups were able to reduce cortisol levels by transporting cortisol back out of the egg by 72 hpf despite the fact they were still immersed in tritiated cortisol (figure 4).

There are several possible explanations for why the inhibited group was able to reduce cortisol levels down to control levels by 72 h. The permeability of the eggs could have decreased such that the proportion of non-inhibited transporters became sufficient to keep cortisol out of the egg [47]. Alternatively, the expression of transporters could have increased [35] or the inhibitor may have degraded over time. Any or all of these explanations may have led to the ability of embryos in the inhibited group to reduce cortisol levels by 72 hpf. Regardless of the reason, these data highlight the capacity of embryos to remove cortisol from the egg. The drop in cortisol levels after an initial uptake suggests that the difference between the cyclosporin A-treated embryos and the control embryos was not owing to developmental effects of cyclosporin A or effects on permeability as these effects would not likely be transient in nature. But as with any pharmacological treatment, we cannot completely rule out the possibility that our results are influenced by unintended side effects. Our transcriptome data suggest that this removal of cortisol from the egg may confer some resistance to an elevation in maternal cortisol exposure, because two different doses of cortisol treatment apparently failed to influence gene transcription within embryos. Additionally, there appears to be a difference in rates of uptake and clearance between two of our studies (figures 2 and 4) despite the fact that eggs were immersed in a water that contained the same concentration of cortisol. Given that wild-caught animals were used in these studies, we speculate that the difference reflects year-to-year variation in egg steroid composition owing either to variation in endogenous steroid content or exposure to environmental chemicals. In fact, environmental chemicals have been shown to decrease the clearance of maternal steroids in other oviparous vertebrates [48]. Importantly, though, both studies show the same pattern: egg steroid levels decrease within 72 h of fertilization. More work is need to examine how endogenous steroids and environmental chemicals might interact to alter clearance rates. Taken together, our results demonstrate that cortisol within the egg at fertilization is removed from the egg, which may ultimately prevent embryonic exposure to maternal cortisol.

There is well-established precedent for the observation that ABC transporters are vital to modulating the exposure of fish to exogenous chemicals [32,35,49]. Indeed, their importance for also regulating exposure to maternal steroids is appreciated in placental mammals [31]. However, this is the first time, to the best of our knowledge, that ABC transporters have been invoked as a mechanism for regulating exposure to maternal steroids in oviparous vertebrates. Reptiles and birds have been shown to modulate embryonic exposure to maternal steroids via *in ovo* metabolism of steroids [14,50,51]. Our studies demonstrate that another important mechanism by which oviparous vertebrate embryos can regulate their exposure to maternal steroids is by an active efflux of steroids from the egg. We hypothesize that active efflux is the preferred method by which fishes (and possibly amphibians) modulate their exposure to maternal steroids, because their eggs are in more direct contact with the external environment (unlike in birds and reptiles). The evolution of the cleidoic egg of birds and reptiles may have resulted in a switch from transporting maternal steroids out of the egg to metabolizing steroids within the egg as transport became less feasible owing to the egg shell.

There is a rich literature documenting the effects of maternal stress on offspring development. Often the hypothesized mechanism linking maternal stress to offspring outcomes is via glucocorticoids, which follows from the observation that cortisol levels in eggs are typically higher following maternal stress [44,52,53], and elevated cortisol has been associated with morphological abnormalities [54], smaller size [55], cardiac dysfunction [56], impaired learning [57] and blunted cortisol responsiveness [58]. However, cortisol levels drop after fertilization [55,58,59] and successful experimental elevation of cortisol levels within eggs requires relatively high doses to achieve small increases [55]. As a whole, this work on cortisol effects suggests that developing embryos attempt to buffer themselves from the potentially detrimental effects of maternal cortisol.

At the same time, there is growing appreciation that maternal experiences, including exposure to certain stressors, might adaptively prepare offspring for certain environments [60–64]. Studies such as this one suggest that such maternal effects might not be mediated by the direct effect of maternal cortisol on offspring. For example, although female sticklebacks increase the cortisol content of their eggs by about 33% following maternal stress during egg production [44], and thousands of genes are differentially expressed between embryos of predator exposed versus unexposed mothers [37], we found that directly exposing embryos to cortisol did not affect the expression of any genes at this same time point. Importantly, maternal stress affected factors other than cortisol content, such as egg size and embryonic metabolic rates [44]. More generally, these studies suggest that equating maternal stress effects to glucocorticoid effects is not always accurate. There are likely a number of scenarios where maternal stress affects offspring without glucocorticoid exposure as well as scenarios where manipulations of glucocorticoid exposure do not mimic maternal stress effects. Studies such as this of the physiological mechanisms underlying maternal stress are

necessary to untangle which effects of maternal stress may or may not be mediated by glucocorticoid exposure.

Going forward, characterizing ABC transporters during development will be important for understanding how organisms may respond to rapidly changing environments. These transporters are likely to not only play a role in how organisms are directly affected by environmental chemicals, but also how environmental perturbations such as invasive species, habitat destruction and climate change might alter embryonic development through maternal steroid exposure. If these transporters serve to buffer embryos, changing environments could increase selective pressures on the capacity of the buffer and influence evolutionary processes. Our results suggest that fish may be in a similar situation to placental vertebrates, birds and reptiles, where the regulation of maternal steroid exposure is dynamic and potentially responsive to environmental conditions. A more detailed understanding of the processes involved in maternal steroid exposure will be vital to deciphering the adaptive significance of maternal steroid effects.

Ethics. All experiments were performed under permission of the University of Illinois Institutional Animal Care and Use Committee (IACUC protocol no. 12118).

Data accessibility. Datasets supporting this article can be found in the electronic supplementary material. RNAseq data can be found at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE71649.

Authors' contributions. R.T.P. and A.M.B. designed the study. R.T.P. conducted the study and analysed the data. S.A.B. analysed the transcriptome data. R.T.P. wrote the manuscript with contributions from S.A.B. and A.M.B.

Competing interests. We have no competing interests.

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