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# Joint maternal and paternal stress increases the cortisol in their daughters' eggs

Whitley R. Lehto and Robin M. Tinghitella

*Department of Biological Sciences, University of Denver, Denver, Colorado, USA*

## ABSTRACT

**Background:** Parental experience with predators can modify survival- and reproduction-related traits of offspring via parental effects. Direct predation risk elevates glucocorticoid concentration in the eggs of females, and so indirect predation risk communicated via parental effects may also affect glucocorticoids in the eggs of daughters. Parents may also change their care patterns under predation risk, which could influence the development of the hypothalamus–pituitary–adrenal axis (stress axis) of offspring, which is responsible for the secretion of glucocorticoids. Therefore, in systems where males make substantial contributions to offspring care, paternal effects may also affect daughters' egg glucocorticoids.

**Question:** Are there predator-induced parental effects (maternal, paternal, or joint parental effects) on the concentration of glucocorticoids in daughters' eggs?

**Organism:** Threespine stickleback (*Gasterosteus aculeatus*) from the Chehalis River, Washington, USA. Freshwater and riverine ecotypes.

**Methods:** We exposed threespine stickleback mothers, fathers, both, or neither to a model predator at developmentally appropriate times using a fully factorial design. Control parents experienced no disturbance. Mothers were exposed to a model predator during egg production and fathers were exposed pre-fertilization and during egg care (but before embryos developed eyes). We then tested the concentration of glucocorticoids in the eggs of daughters using an enzyme-linked immunosorbent assay (ELISA).

**Results:** Daughters of predator-exposed parents (both parents exposed to model predator) had higher glucocorticoid concentrations in their eggs than daughters of control, unexposed parents. Daughters of predator-exposed mothers-only and predator-exposed fathers-only did not differ from controls or jointly predator-exposed parents. Therefore, predator-induced maternal and paternal effects may cumulatively impact the gametes of their daughters, suggesting a mechanism through which predation risk may indirectly influence the next generation (grand-offspring).

*Keywords*: cortisol, maternal effect, parental effect, paternal effect, predator, threespine stickleback.

Correspondence: W.R. Lehto, Department of Biological Sciences, University of Denver, 2190 E. Iliff Ave., Room 102, Denver, CO 80210, USA. email: lehtowhi@gmail.com

## INTRODUCTION

The stressors that parents experience can impact the interactions they have with their offspring. Under stressful conditions, parents can alter the developmental and rearing environment of their offspring through their own physiological responses to stress (i.e. hormones) or by changing their parental care regimes (Badyaev and Uller, 2009; Crean and Bonduriansky, 2014). Either of these can result in parental effects, or variation in offspring phenotypes attributable to variation in parent–offspring interactions rather than differences in parents' genotypes. Parental effects allow parents to indirectly 'communicate' their experience with environmental challenges to their offspring (Sheriff *et al.*, 2010; Sheriff and Love, 2013), in some cases resulting in adaptive offspring responses that parallel the effects of direct exposure to the same environmental stressor (Mousseau and Fox, 1998; Storm and Lima, 2010; Burgess and Marshall, 2014).

The stress mothers experience in their environment can change the concentration of glucocorticoid stress hormones their offspring are exposed to during development in egg-laying and placental/gestating species (Love *et al.*, 2013). Glucocorticoids (including cortisol) are steroid hormones found in vertebrates that are implicated in metabolism and stress responses (Sapolsky *et al.*, 2000; Bonier *et al.*, 2009). The hypothalamus–pituitary–adrenal (or interrenal) axis (HPA axis) is the endocrine axis responsible for secretion of glucocorticoids. Exposure to elevated maternal cortisol can influence the formation of the HPA axis in offspring (Sapolsky *et al.*, 2000), generating variation in the responsiveness of offspring to stress by reducing their ability to buffer stress or preventing them from responding to stress when it would be adaptive to do so (Sapolsky *et al.*, 2000; Love *et al.*, 2013). Typically, the secretion of glucocorticoids increases with exposure to a stressor, and then decreases as the stressor is mitigated (e.g. via a physiological or behavioural response) through negative feedback when the glucocorticoids bind to glucocorticoid receptors and mineralocorticoid receptors in the hippocampus (Liu *et al.*, 1997; Sapolsky *et al.*, 2000; Matthews, 2002). Elevated glucocorticoid exposure during development is thought to decrease the number of glucocorticoid receptors and mineralocorticoid receptors (Liu *et al.*, 1997; Sapolsky *et al.*, 2000; Love *et al.*, 2013); therefore, in animals exposed to elevated glucocorticoids during development, glucocorticoids secreted in response to stress will circulate for longer, producing a stressed phenotype even in the absence of a stressor (Sheriff *et al.*, 2010) or a reduced sensitivity to stress (Auperin and Geslin, 2008). Elevated glucocorticoids during development have effects on many offspring traits, including decreased activity and increased anxiety in zebrafish (Best *et al.*, 2017) and slowed growth and higher corticosterone in Japanese quail (Hayward and Wingfield, 2004).

Variation in parental care also impacts development of the HPA axis (Liu *et al.*, 1997; Francis and Meaney, 1999). In rats, for instance, cross-fostered offspring that receive less maternal care show decreased expression of glucocorticoid receptors, demonstrate low maternal care themselves, and display more fearful behaviours; thus, maternal care and stress responses depend on non-genomic maternal effects (Francis *et al.*, 1999). Maternal effects have been studied more often than paternal effects, but in many species (e.g. many birds and fish), fathers and/or both parents make substantial contributions to offspring, making both maternal and paternal effects important determinants of offspring phenotypes. In threespine stickleback, fathers perform all parental care, and offspring reared without a father display more anxiety-related behaviours than offspring that receive paternal care (McGhee and Bell, 2014). In organisms with biparental care, the removal of one parent also seems to impact stress-related hormones and behaviours; for example, zebra finches reared without a mother

display higher concentrations of corticosterone relative to those reared by both parents (Banerjee and Aterberry, 2012), and California mice have both decreased survival and increased stress-related behaviours when deprived of paternal care (Glasper *et al.*, 2018). Together, the studies on zebra finches and California mice, which deprived offspring of care from one parent only with dramatic effects, point to the need to examine maternal, paternal, and joint parental effects in systems with large biparental contributions to offspring development. This would reveal whether parental contributions are independent, act in the same or different direction, and interact with one another. Additionally, paternal effects underlain by changes in sperm characteristics, though historically under-appreciated, have the potential to influence offspring HPA axis regulation (Rodgers *et al.*, 2013) and survival (Crean *et al.*, 2013).

Furthermore, many studies that manipulate parental stress or contributions (e.g. artificial exposure to glucocorticoids in early development or parental absence) are not necessarily derivative of the ecological challenges that parents face. Predation risk is a ubiquitous ecological stressor known to influence the glucocorticoids of mothers (Giesing *et al.*, 2011; Monclús *et al.*, 2011; Love *et al.*, 2013; Sopinka *et al.*, 2016) and the care parents provide to offspring (Magnhagen, 1992; Stein and Bell, 2012; Ghalambor *et al.*, 2013; Vitousek *et al.*, 2014). There are numerous examples of predator-induced parental effects on offspring morphology (Agrawal *et al.*, 1999; Stein and Bell, 2014), anti-predator behaviour (Storm and Lima, 2010), learning (Roche *et al.*, 2012), life history (Walsh *et al.*, 2015), and reproduction (Lehto and Tinghitella, in review).

Our study system, the threespine stickleback (*Gasterosteus aculeatus*), allows us to compare the separate and combined impacts of maternal and paternal effects on offspring traits. Threespine stickleback mothers and fathers make independent contributions to offspring at different stages of development. Maternal and paternal experience at the pre-fertilization and post-fertilization stages could contribute to restructuring the HPA(I) axis of offspring. Female stickleback produce energetically expensive eggs, but provide no parental care. Direct predation risk to mothers elevates glucocorticoid concentration in their eggs (Giesing *et al.*, 2011), which has been interpreted as an adaptive response to parental stress because juvenile offspring of predator-exposed females (during egg production) exhibit tighter shoaling behaviour, which is an adaptive strategy in a predator-rich environment [but see McGhee *et al.* (2012) and Roche *et al.* (2012) for maladaptive maternal effects on adult offspring antipredator behaviour and learning, respectively, in the same study system]. After a female deposits a clutch of eggs in a male's nest, the male performs all parental care for stickleback eggs (oxygenation, removing rotten eggs and debris, territory defence) and fry (chasing and retrieving fry that stray from the nest and continued territory and offspring defence) for 3–15 days (Wootton, 1984; Tulley and Huntingford, 1987). Paternal care behaviour is also modified (decreased) by direct exposure to predators (Stein and Bell, 2012), and fathers exposed to predators during parental care produce offspring that are smaller at sexual maturity (presumably adaptive in predator-rich environments) and daughters with higher circulating cortisol (Stein and Bell, 2014). Therefore, both maternal and paternal stress (and their combined impacts) have the potential to alter offspring stress responses in this system, although stress-induced maternal and paternal effects on offspring stress (neurobiology, physiology, and behaviour) are rarely addressed in the same study (but see Yehuda *et al.*, 2014).

In a previous study, we assessed the independent effects of maternal and paternal predator-exposure as well as joint parental predator-exposure on daughters' behaviour (Lehto and Tinghitella, in review). In that study, joint parental effects impacted the mating behaviour of daughters differently than maternal and paternal predator-exposure alone. Specifically, predator-induced maternal and paternal effects led daughters to relax or reverse their

typical preferences for conspicuous, colourful males, whereas daughters from predator-exposed parents (joint parental effects) preferred conspicuous mates (similar to the preferences of unexposed control parents). Importantly, this pattern means that we cannot assume that maternal and paternal predator-exposure are additive. The finding also underscores the importance of comparing maternal, paternal, and joint parental effects in study systems that facilitate such work. Here, in a *post hoc* investigation, we address whether maternal, paternal, and joint parental stress via predator-exposure influences the glucocorticoids, specifically cortisol, that daughters have in their eggs, which may (1) inform us about the relative and combined impacts of predator-induced parental effects on daughters' stress-related physiology, and (2) provide a window into the manner in which parental effects may be passed through daughters' gametes to the next generation.

Stickleback can provide an opportunity to probe the effects of parental stress via pre-fertilization/early embryonic exposure to maternal glucocorticoids and pre-fertilization (sperm) effects and embryonic/post-hatching paternal care on offspring physiology and stress response. In this study, we exposed mothers, fathers, both, or neither to a stressor (a model predator) using a fully factorial design. Given that direct predation risk to stickleback mothers elevates the cortisol found in their eggs (Giesing *et al.*, 2011) and that parental effects are often predicted to modify offspring traits in parallel with direct effects (Mousseau and Fox, 1998; Uller, 2008), we hypothesized that parental predator-exposure would elevate the cortisol detected in the eggs of daughters. If so, the indirect effects stemming from the predation risk to parents on egg cortisol should parallel the direct effects of predation on egg cortisol. Furthermore, we hypothesized that both maternal and paternal effects may elevate daughters' egg cortisol but to varying degrees due to differences in developmental contributions of mothers and fathers, while joint parental effects may cumulatively increase daughters' egg cortisol.

## MATERIALS AND METHODS

### Field collection sites and animal husbandry

We collected reproductively ready adult, freshwater stickleback from the Chehalis River, Washington, USA (46°56′47.4″N, 123°38′30.5″W and 46°58′46.8″N, 123°28′41.4″W) and transferred them to the University of Denver in summer 2015 for laboratory crosses. Temperature and photoperiod conditions tracked those occurring in southwest Washington to simulate breeding conditions throughout the season. We housed parental fish in visually isolated, same-sex holding tanks (110 L, 77 cm × 32 cm × 48 cm) at densities of no more than 30 fish per tank and fed them a mixture of bloodworms and *Artemia* daily scaled for the number of individuals per tank approaching *ad libitum*.

### Parental predator-exposure and laboratory crosses

To assess the influence of maternal, paternal, and joint parental effects on daughters' egg cortisol, we used a complete factorial cross design in which neither parent, the mother only, the father only, or both parents were predator-exposed to produce four treatments: control ($n = 15$ among four families), predator-exposed mother ($n = 16$ among five families), predator-exposed father ($n = 16$ among five families), and predator-exposed parents ($n = 20$ among five families).

We exposed wild-caught adult males and females to a model predator common to Washington state rivers (Jewel Bait Co. © Sculpin Hypertail), which resembles shorthead sculpin (*Cottus confusus*) during the phases of development at which each sex makes an important contribution to offspring: for females, during egg formation, and for males, pre-mating and during egg care. More specifically, we randomly assigned adult females to be predator-exposed or unexposed and housed them in two separate holding tanks at equal densities. Unexposed females were left undisturbed. To produce predator-exposed females, we moved the model predator through their holding tank for 30 seconds each day at a random time of day during the period that females were developing a clutch of eggs (following Giesing *et al.*, 2011; McGhee *et al.*, 2012; Roche *et al.*, 2012). The stickleback may have responded to visual cues, physical cues (movement of water and tank substrate), or cues from conspecifics resulting from the predator model: we were interested in predation risk as a representative ecological stressor and in capturing any and all consequences.

Each experimental male was placed in his own nesting tank (76 L, 61 cm × 30 cm × 41 cm) and left undisturbed while building a nest in a tray of sand. When a female was fully gravid, we randomly assigned her to a male with a readied nest. We also then randomly assigned the male to be either predator-exposed or unexposed. Predator-exposed males had a predator model move through their nesting tank for 30 seconds, 15 minutes before the courtship trial to elicit pre-fertilization paternal effects that may stem from predation risk and to simulate ecologically relevant parental predator-exposures (i.e. fathers are likely to face predation risk before mating and during parental care).

Once the parents were prepared for the cross, we used standardized 'no-choice' mating trials (following Nagel and Schluter, 1998; Head *et al.*, 2009; Tinghitella *et al.*, 2013) to produce offspring. We gently introduced the female (mother) into the male's (father's) tank through a tube with a false floor. After a two-minute acclimation in the tube, the mating pair were allowed up to 20 minutes to spawn. At the end of a successful cross, we returned the females to holding tanks. Males remained in their nesting tanks to resume paternal care. A given female or male was allowed up to three no-choice trials to produce a successful cross, but no fish was used more than once in a successful cross. Finally, predator-exposed males underwent a second post-mating predator exposure for 2 minutes on day 3 of egg care (following Stein and Bell, 2014) when the embryos were yet to have fully developed eyes (Swarup, 1958). Unexposed males were left undisturbed, both pre-mating and during parental care. Predator-exposed males reduced their number of nest visits by 20% and reduced the time they spent fanning their nests by 37% when mated with predator-exposed females (Lehto and Tinghitella, in review). Following no-choice courtship trials and mating, we raised the offspring of crosses to sexual maturity (approximately one year of age), housing them by family. Stickleback fry were fed live *Artemia* nauplii and juveniles were fed a mixture of live *Artemia* and prepared bloodworms daily. Offspring experienced no direct predation cues.

### Daughters' egg size, egg number, and egg cortisol

When daughters reached adulthood a year later and became gravid, we assessed their behaviour in no-choice mating trials for a study of parental effects on mate preferences and mate choice (Lehto and Tinghitella, in review). When daughters were gravid, we massed them and photographed them while bearing eggs to obtain body length via FIJI (from the anterior extent of the mouth to the caudal extent of the tail), scaled using a millimetre ruler placed in the photograph. Immediately after daughters underwent their mating trial, we stripped

their eggs, and massed them again. We counted the eggs to assess any impacts of parental predator-exposure on egg number and then stored them in ethanol. We determined clutch weight (mass with eggs – mass without eggs) to ultimately determine egg size (clutch weight/number of eggs), as daughters' egg size may also change with parental predator-exposure given the direct effects of predation on egg size (Giesing *et al.*, 2011). We measured egg cortisol content using an enzyme-linked immunosorbent assay (ELISA; Enzo Life Sciences Cat. No. ADI-900-071). We tested daughters' egg cortisol concentrations in duplicate. We prepared each sample (without extraction) by removing five eggs from a daughter's full clutch and homogenizing them in 100 $\mu$L of 1 × TBS with a microtube homogenizer and pestle. We read the absorbance of each sample using a BioTek Synergy HTX Multi-Mode Reader at 405 nm using area scanning (we obtained a mean optical density value for 25 readings spread within each single well). To calculate the amount of cortisol in our samples, we used a standard curve, fitting a 4-parameter logistic (4PL) curve to the standard wells using Gen5 v.3.0, following the kit manual. All measured egg cortisol values were above the minimum kit sensitivity. We then obtained a mean egg cortisol content value for each daughter, which was used in statistical analyses.

### Statistical analysis

We tested for maternal, paternal, and joint parental effects (treatment as a fixed effect) on egg cortisol content, egg size, and number of eggs using linear mixed models (LMMs). We included female length as a covariate in the models because female size is an established predictor of egg size and number in fish (Wootton, 1973; Morita and Takashima, 1998; Heinimaa and Heinimaa, 2004), and family nested within treatment as a random effect. Mean egg cortisol concentrations were not normally distributed and were thus ln-transformed. To account for potential variation in egg cortisol stemming from females' experience with male mates during courtship, we also included male ID in the model testing for parental effects on egg cortisol content. We reduced each full model by sequentially removing least-significant covariates and then refit each model. We performed all LMMs using *lmer* in the lme4 package (Bates *et al.*, 2017) and effects testing using likelihood ratio tests with *mixed* in the afex package (Singmann *et al.*, 2018) in R v.3.5.1 (RStudio v.0.99.903).

## RESULTS

We found parental effects on the cortisol content of eggs of daughters whose parents experienced predation risk (Table 1; Fig. 1). When both parents were predator-exposed, daughters' eggs had 40% more cortisol than those of unexposed parents (Tukey's HSD: estimate ± S.E., 0.34 ± 0.12, $z = 2.84$, $P = 0.02$; Fig. 1; effect size calculated using back-transformed LS means: LS means ± S.E.: control, 185 ± 1.11 pg/mL; predator-exposed parents, 253.15 ± 1.09 pg/mL). Daughters' egg cortisol did not differ significantly among other pairwise treatment comparisons. That is, the egg cortisol of daughters who had only one parent who was predator-exposed (mother or father) did not differ from one another, from control daughters, or from daughters whose parents both experienced predator-exposure. We found no evidence for parental effects (maternal, paternal, or both) on egg size or number of eggs (Table 1). Female length was not a significant covariate on egg cortisol ($P = 0.35$) or egg size ($P = 0.36$).

**Table 1.** LMM effects on daughters' eggs: cortisol content, size, and number

| Response variable | Effect | $\chi^2$ | *df* | *P* |
|---|---|---|---|---|
| ln(egg cortisol) (pg/mL) (Male ID = random) | Treatment | 9.02 | 3 | 0.03 |
| Egg size (mg) | Treatment | 3.64 | 3 | 0.30 |
| Number of eggs | Treatment | 3.25 | 3 | 0.35 |
| | Female length | 26.83 | 1 | <0.0001 |

*Note*: Treatment refers to parental predator-exposure regime (neither parent, single parent, or both parents). All models included family nested within treatment as a random effect.

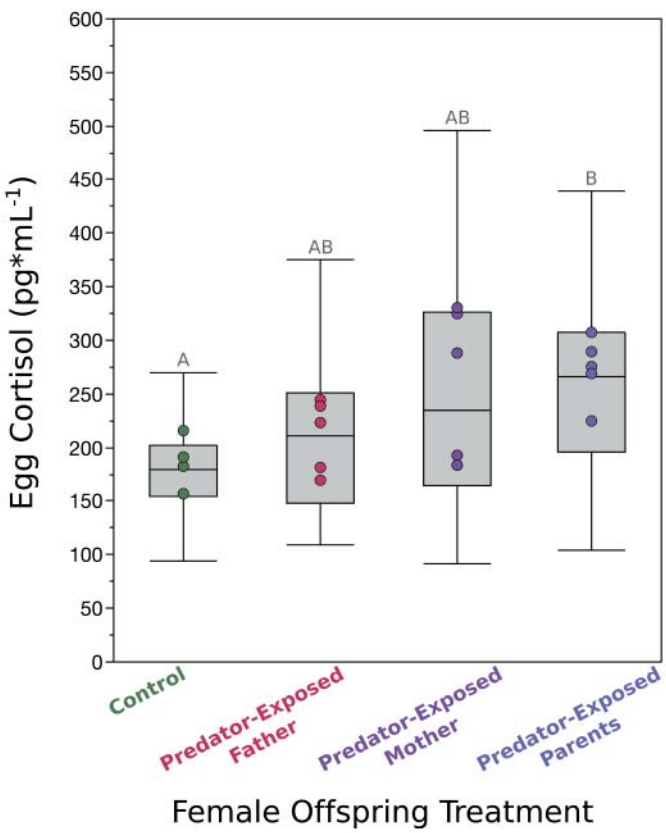


**Fig. 1.** Predator-induced parental effects increase the cortisol concentration in daughters' eggs. Box-plots show the egg cortisol of daughters when neither parent (control), their mother (predator-exposed mother), their father (predator-exposed father), or both parents (predator-exposed parents) experienced predation risk during egg production (mothers) or parental care (fathers). Egg cortisol values used in statistical models were ln-transformed. Letters above box plots show significant differences among treatments (Tukey's test, $\alpha = 0.05$). Dots within each treatment represent family means.

## DISCUSSION

Direct exposure to predation risk in stickleback females increases the cortisol content in their eggs (Giesing *et al.*, 2011). Here, we demonstrate for the first time that predation risk to parents also modifies the cortisol content of their daughters' eggs through parental effects, providing a potential mechanism for transgenerational responses to environmental stress. Daughters of parents who were both exposed to a model predator (joint parental effects) had eggs containing 40% more cortisol than control daughters whose parents were left undisturbed. Under direct predation risk, mothers' eggs contained 35% more cortisol than unexposed mothers (Giesing *et al.*, 2011). The magnitude of difference in egg cortisol between daughters of predator-exposed parents and daughters of control parents is thus comparable to that which stems from direct predator-exposure. Therefore, as hypothesized, parental effects on daughters' egg cortisol (perhaps established epigenetically during development) parallel the plastic effects of direct predator-exposure on mothers' eggs. In other study systems, exposure to increased cortisol during development yields offspring with 'stressed' phenotypes, reflected in decreased activity levels, increased anxiety, or slow growth (Hayward and Wingfield, 2004; Best *et al.*, 2017). We do not yet know if the parental effects on daughters' egg cortisol uncovered here are representative of daughters' baseline cortisol concentrations or if this variation in cortisol is sufficient in magnitude to directly impact stress responses (adaptively or not) in daughters or in their offspring. Yet, we do find evidence for behavioural differences consistent with adaptive stress responses in the same daughters used in this study (Lehto and Tinghitella, in review), suggesting underlying differences in physiology. It is also possible that daughters' egg cortisol was established, perhaps epigenetically (Ho and Burggren, 2010), during development operating, at least in part, independently of plasma cortisol concentrations. An experimental design incorporating measurements of direct predation risk on maternal plasma and egg cortisol with maternal effects on offspring plasma and egg cortisol would further elucidate the mechanisms underlying parental effects on stress hormones and associated variation in behaviour (of offspring and grand-offspring).

Direct predation risk has been shown to increase egg size in threespine stickleback (Giesing *et al.*, 2011), though we did not find predator-induced parental effects on egg size in this study. It is not uncommon to find that direct effects on parental phenotypes are not similar in direction or magnitude to parental effects on offspring phenotypes (Walsh *et al.*, 2015), especially when the environment of offspring does not reinforce the parental environment [for instance, when the offspring environment is predator-free while the parents' was predator-rich; i.e. intergenerational phenotype 'wash-out' (Burggren, 2015)]. Alternatively, the effects of direct predator-exposure and predation risk on egg size and egg cortisol simply may not parallel the indirect effects of transgenerational parental effects. However, methodological differences between studies may also contribute to differences between the effects of direct predator-exposure and predation risk of parents on egg size. Here, we counted egg number directly, calculating egg size on the basis of that and the whole clutch mass, whereas in previous work egg number was estimated based on average egg mass and overall clutch mass (Giesing *et al.*, 2011).

Our experimental design and the threespine stickleback study system provided us with a unique opportunity to examine the relative importance of and joint impacts of maternal and paternal predator-exposure on daughters' egg cortisol. We found that it was only when both parents were exposed to the predator model that daughters' eggs contained significantly more cortisol than those of unexposed parents. That is, it appears that maternal

and paternal predator-exposure alone do not induce substantial variation in daughters' egg cortisol. One possible explanation is that males can detect predator-exposure of their mates and modify paternal care in ways that buffer effects of maternal predator-exposure [i.e. through the process of social buffering (Faustino *et al.*, 2017)]. Although predator-exposure reduces paternal care (Lehto and Tinghitella, in review), that alone was not sufficient to change daughters' egg cortisol (this study). Stickleback males can detect the predator-exposure history of their female mates using both visual and olfactory cues (Dellinger *et al.*, 2018). Fathers in this study reduced their care when mated with predator-exposed females (Lehto and Tinghitella, in review); thus it is only when both developmental exposure to cortisol (Giesing *et al.*, 2011) *and* paternal care (Stein and Bell, 2012) are changed through joint parental predator-exposure that we find detectable effects on daughters' egg cortisol. Upon visualization of our data, however, it is clear that there is considerable variation in the cortisol concentrations of daughters from predator-exposed mothers. This prompted us to conduct a power analysis. Our power to detect an effect of maternal predator-exposure on daughters' egg cortisol was indeed lower than our power to detect an effect of joint parental predator-exposure [46.7% vs. 67.8%; power analysis performed using the *powerSim* function with 1000 simulations in the simr package in R (Green *et al.*, 2018)]. With a modest increase in sample size, then, we might find that maternal effects, both when the mother alone and when both parents are predator-exposed, are the most critical determinant of daughters' egg cortisol. Such an effect might stem from exposure to maternal cortisol at the earliest stages of development. We encourage future work in biparental care systems, in particular to illuminate our understanding of and disentangle the relative impacts of maternal and paternal care and the critical periods at which developmental environments influence offspring phenotypes.

Parental effects have been of considerable interest recently because of their potential to facilitate rapid and transgenerational responses to changing environments (Ghalambor *et al.*, 2015; Chirgwin *et al.*, 2018). We have uncovered parental effects on glucocorticoids in the gametes of daughters whose parents were exposed to an ecologically relevant stressor. That we find effects on gametes suggests that there may also be grandparental effects of predator-exposure. Increased developmental glucocorticoid exposure in the F2 generation (grand-offspring) may impact a variety of physiological and behavioural processes, many of which, if adaptive, could allow organisms to respond to stressors in their environment. It would be fruitful to link parental effects (separate and joint) on glucocorticoids such as cortisol to variation in offspring and grand-offspring stress responses that could ultimately be selected upon in new, challenging environments.

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