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# Proximate and Ultimate Consequences of Stressed-Induced Maternal, Paternal, and Joint Parental Effects in a Changing World

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## Abstract

Parental experience can alter the developmental and rearing environments of offspring, resulting in parental effects on offspring traits. I addressed the consequences of stress-induced maternal, paternal, and joint parental effects from both ultimate (ecological/evolutionary) and proximate (physiological/epigenetic) perspectives. I used a full-factorial design in which threespine stickleback (*Gasterosteus aculeatus*) mothers, fathers, both, or neither were exposed to a model predator at developmentally appropriate times to test for predator-induced maternal, paternal, and joint parental effects on daughters' mating behavior and egg glucocorticoids (stress hormones) and on offspring gene expression. Maternal and paternal predator exposure independently yielded daughters who preferred less conspicuous mates with duller nuptial coloration and who courted less vigorously, relaxing (paternal) or reversing (maternal) typical preference for conspicuous males. The combined effects of maternal and paternal predator exposure were not cumulative; when both parents were predator-exposed, single-parent effects on daughters' mate preferences were reversed. Therefore, parental effects may alter the direction of sexual selection. I tested the concentration of glucocorticoids, specifically cortisol, in the eggs of daughters post-mating trial using an enzyme-linked immunosorbent assay (ELISA). 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 control or jointly predator-exposed parents' daughters. Therefore, predator-induced parental effects impact the gametes of their daughters, suggesting a mechanism through which predation risk may indirectly influence the next generation (grand-offspring). Finally, offspring gene expression varied with the source of parental effects: maternal and paternal effects on offspring gene expression were similar to each other, but each was different from joint parental effects. There were no differences in offspring gene expression when parent and offspring matched and mismatched (when offspring did or not experience direct predation risk themselves), perhaps because of the animals' age at direct exposure and the specific method of predator-exposure used in this study. Maternal and paternal effects appear to be underlain by different epigenetic changes that yield independent, but perhaps additive, variation to offspring gene expression that could have an array of impacts on offspring phenotypes. Thus, stress-induced maternal, paternal, and joint parental effects may potentiate rapid transgenerational responses to novel and changing environments.



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immunosorbent assay (ELISA). 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 control or jointly predator-exposed parents' daughters. Therefore, predator-induced parental effects impact the gametes of their daughters, suggesting a mechanism through which predation risk may indirectly influence the next generation (grand-offspring). Finally, offspring gene expression varied with the source of parental effects: maternal and paternal effects on offspring gene expression were similar to each other, but each was different from joint parental effects. There were no differences in offspring gene expression when parent and offspring matched and mismatched (when offspring did or not experience direct predation risk themselves), perhaps because of the animals' age at direct exposure and the specific method of predator-exposure used in this study. Maternal and paternal effects appear to be underlain by different epigenetic changes that yield independent, but perhaps additive, variation to offspring gene expression that could have an array of impacts on offspring phenotypes. Thus, stress-induced maternal, paternal, and joint parental effects may potentiate rapid transgenerational responses to novel and changing environments.

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*Ethics statement:* The experiments of this dissertation research were approved by the Institutional Animal Care and Use Committee of the University of Denver (protocol no.: 472426-9). Scientific collection (15-198) and transfer (7149-05-15) permits were approved by Washington Department of Fisheries and Wildlife.

# TABLE OF CONTENTS

# LIST OF FIGURES

# Chapter One

## Predator-induced maternal and paternal effects independently alter sexual selection

*Keywords:* parental effect, maternal effect, paternal effect, sexual selection, behavior, mate choice

**ABSTRACT**

Parental experience alters survival-related phenotypes of offspring in both adaptive and non-adaptive ways, yielding rapid transgenerational fitness effects. Yet, fitness comprises survival and reproduction, and parental effects on mating decisions could alter the strength and direction of sexual selection affecting long-term evolutionary trajectories, maintenance of species boundaries and the generation of biodiversity. We used a full factorial design in which threespine stickleback (*Gasterosteus aculeatus*) mothers, fathers, both, or neither were exposed to a model predator at developmentally appropriate times to test for predator-induced maternal, paternal, and joint parental effects on daughters' mating decisions. We tested the mate choices of adult daughters in no-choice trials with wild-caught males who had varied sexual signals. Maternal and paternal predator exposure independently yielded offspring who preferred less conspicuous mates with duller nuptial coloration and who courted less vigorously, relaxing (paternal) or reversing (maternal) typical preference for conspicuous males. The

combined effects of maternal and paternal predator exposure were not cumulative; when both parents were predator-exposed, single-parent effects on mate preferences were reversed. Thus, we cannot assume that maternal and paternal effects additively combine to produce "parental" effects. Stress-induced parental effects on reproductive decisions may potentiate rapid transgenerational responses to novel and changing mating environments.

## INTRODUCTION

Mate choice is the gatekeeper of evolutionary change. Individuals who successfully secure mates (and kin with whom they share genes) leave copies of their genes in future generations. Mating preferences and decisions are also notoriously plastic; they respond strongly to changes in the chooser's internal condition and external ecological and social experience (reviewed in (Cotton, Small, & Pomiankowski, 2006; Jennions & Petrie, 1997; Rosenthal, 2017)). Predation is one ubiquitous stressor that dramatically alters ecological and social interactions, including those between parents and their offspring. Such 'parental effects' are non-genomic ways in which parents' experience can influence offspring traits. Much recent attention has focused on the potential for parental effects to facilitate rapid inter- and transgenerational responses to novel and changing environments (Burton & Metcalfe, 2014; Kokko et al., 2017). Emphasis, however, has been on how parental effects that anticipate the parental environment enhance offspring *survival* characteristics (Beaty et al., 2016; Giesing, Suski, Warner, & Bell, 2011; McGhee & Bell, 2014; Roche, McGhee, & Bell, 2012; Stein & Bell, 2014; Storm & Lima, 2010; Walsh, Cooley IV, Biles, & Munch, 2015). Whether environmentally-induced parental effects extend through development to also affect offspring *reproductive decisions* remained untested, until now. Yet, parental effects on reproduction are as important, or more so, than those on survival because mating decisions directly impact the maintenance of species boundaries and generation of biodiversity. Moreover, the fitness consequences of (often epigenetic) parental effects can be surprisingly long-lived, lasting for 14+ generations in some systems (Houri-Zeevi & Rechavi, 2017; Klosin, Casas, Hidalgo-Carcedo, Vavouri, & Lehner, 2017; Shama &

Wegner, 2014), so parental effects on mate choice could shift long-term evolutionary trajectories. Here, we test whether ecologically relevant variation in parental experience translates to changed mating preferences of progeny via parental effects.

We have known for decades that mating behavior responds strongly to direct experience (Candolin, 1998; Endler, 1983; Hedrick & Dill, 1993). More recently, a rich literature has amassed uncovering vast experience-mediated adjustment of mating preferences and choice, and how this sometimes adaptive regulation of behavior impacts fitness (e.g. (Bailey & Zuk, 2008; Chaine & Lyon, 2008; Fowler-Finn & Rodríguez, 2012; Lynch, Rand, Ryan, & Wilczynski, 2004; R. M. Tinghitella, Weigel, Head, & Boughman, 2013)). Whether choosers are 'stringent or permissive' (Rosenthal, 2017) as a consequence of experience, and with respect to which courter traits, changes the strength and direction of sexual selection. Here, we advance the field by asking whether mating preferences and decisions are also influenced by indirect information gleaned through interactions with parents. Given that standing variation in parents' sexually selected traits affects the reproductive behavior of offspring through imprinting (Kozak, Head, & Boughman, 2011), learning (Verzijden & ten Cate, 2007), and parental care (Cameron, 2011; Cameron, Fish, & Meaney, 2008), we hypothesize that environmental variability that alters parents' interactions with offspring may also change reproductive characteristics of offspring through parental effects.

Many animals experience predation risk during mating; under high predation risk, females often shift their mate preferences and choices to favor less conspicuous mates (Jennions & Petrie, 1997; Lima, 1998). Thus, direct predation risk changes the course of sexual selection and population differentiation (Kozak & Boughman, 2015; Maan &

Seehausen, 2011). Faced with predation, parents sometimes alter provisioning and care for their offspring (Ghalambor, Peluc, & Martin, 2013; Magnhagen, 1992; Smith & Wootton, 1995) providing an epigenetically-mediated mechanism for indirect effects on survival-related traits of offspring (antipredator morphology (Beaty et al., 2016; Stein & Bell, 2014) and behavior (Giesing et al., 2011; McGhee, Pintor, Suhr, & Bell, 2012; Storm & Lima, 2010), learning (Roche et al., 2012), and life history (Walsh et al., 2015)). By extension, parents may communicate their experience (Jablonka, 2002) to offspring before birth or during rearing in ways that alter offspring reproductive characteristics. Some recent evidence from birds and rats demonstrates the types of changes parental effects might induce in mating traits. For instance, stressful post-natal rearing environments (larger clutches) lead to less pronounced adult mate preferences (Holveck & Riebel, 2010; Riebel, Naguib, & Gil, 2009) and egg laying order changes the strength of female preferences (Burley & Foster, 2004) and choosiness (Forstmeier, Coltman, & Birkhead, 2004) in zebra finches. Female descendants of rats exposed to fungicides also have stronger preferences for unexposed mates than do descendants of control rats (Crews et al., 2007).

Further, in many birds, fish, and insects, both mothers and fathers make important contributions to offspring development and success, yet inter- and transgenerational effects of fathers have been largely overlooked (Crean & Bonduriansky, 2014; Crews, Gillette, Miller-Crews, Gore, & Skinner, 2014). *Maternal* and *paternal* effects have also rarely been addressed in a single study, and the two are often assumed to act in the same direction (e.g. (Head, Berry, Royle, & Moore, 2012)) and/or to have cumulative effects (e.g. (Hunt & Simmons, 2000)).

We capitalize on an ideal study system that allows us to compare the separate and combined impacts of maternal and paternal effects on offspring reproductive decisions. Threespine sticklebacks (*Gasterosteus aculeatus*) 1) have well-characterized mating preferences, 2) provide independent maternal and paternal contributions to offspring development, and 3) influence offspring survival-related traits through parental effects. First, in the breeding season, most male threespine sticklebacks, including the marine ancestors of the riverine fish we study here, develop a bright red throat that extends from the mouth to the pelvic spines, and contrasts with a blue eye (Flamarique, Bergstrom, Cheng, & Reimchen, 2013). Females strongly prefer males with extensive and intense red throat and blue eye coloration (Baube, Rowland, & Fowler, 1995; Boughman, 2001; Boughman, Rundle, & Schluter, 2005; Milinski & Bakker, 1990; Rowland, 1994), characteristics that are conspicuous to predators (Johnson & Candolin, 2017). The red throat signals physical condition, parasite resistance, nest defense, and mating success (Albert, Millar, & Schluter, 2007; Bakker & Milinski, 1993; Boughman, 2001; Smith, Barber, Wootton, & Chittka, 2004), so females gain both direct and indirect benefits from preferred males. Second, mother and father sticklebacks each make substantial, but distinct, contributions to offspring development. Mothers produce energetically expensive eggs and then choose amongst males who have secured territories and built nests. After a sequence of courtship interactions, if the female finds the male acceptable for mating, she enters the nest to deposit a clutch of eggs. Males then assume all parental care for eggs (oxygenation, removing rotten eggs and debris, and territory defense) and fry (chasing and retrieving of fry that stray from the nest and continued territory and offspring defense) for 3 to 15 days (Tulley & Huntingford, 1987; Wootton, 1984). Third,

both maternal and paternal experiences with predators influence the survival-related traits of stickleback offspring. Maternal predator-exposure reduces offspring learning speed (Roche et al., 2012) and hinders the anti-predator behavior of adult offspring (McGhee et al., 2012), but enhances juvenile shoaling anti-predator responses (Giesing et al., 2011). Paternal predator-exposure alters paternal care behavior leading to offspring morphology and activity levels that are consistent with direct experience with predators (Stein & Bell, 2014), and offspring reared without a father have higher anxiety behavior potentially owing to epigenetic changes in methylation (McGhee & Bell, 2014).

Mating-related traits of both males and females respond plastically and evolutionarily to direct predation risk in predictable ways: males often display less conspicuous ornaments and courtship behaviors (Candolin, 1998; Endler, 1983; Magnhagen, 1991), and females reduce interest in conspicuous mates (Candolin, 1997; Endler, 1983; Hedrick & Dill, 1993; Wong & Rosenthal, 2006). If parental predator exposure influences offspring reproduction, we expect adaptive parental effects on daughters' preferences to act in the same direction, relaxing sexual selection. Here, we demonstrate that maternal and paternal effects independently reduce female interest in mating, change the shape of daughters' preference functions and their mate choices. Thus, we have found that parental effects can change sexual selection. Further, while maternal and paternal predator exposure independently shifted daughters' mating preferences from more conspicuous to less conspicuous mates, the combined effects of maternal and paternal predator exposure were not cumulative; when both parents were predator-exposed the direction of sexual selection was reversed compared to when either parent was exposed alone.

## MATERIALS AND METHODS

*Parental Predator-Exposure and Laboratory Crosses*

To assess the influence of maternal, paternal, and joint parental effects on offspring mating behavior, 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=16 from 4 families, 2-5 offspring per family), predator-exposed mother (n=17 offspring from 5 families, 2-5 offspring per family), predator-exposed father (n=17 offspring from 5 families, 3-5 offspring per family), and predator-exposed parents (n=20 from 5 families, 3-5 offspring per family) (Figure 1.1). We collected reproductively ready adult sticklebacks from the Chehalis River, WA (N46°56'47.4" W123°38'30.5"; N46°58'46.8" W123°28'41.4") and transferred them to the University of Denver in summer 2015 for laboratory crosses. Temperature and photoperiod conditions in the lab 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 x 32 cm x 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*.

In the lab, we randomly assigned adult females and males to be predator-exposed or unexposed. To simulate predator exposure, 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

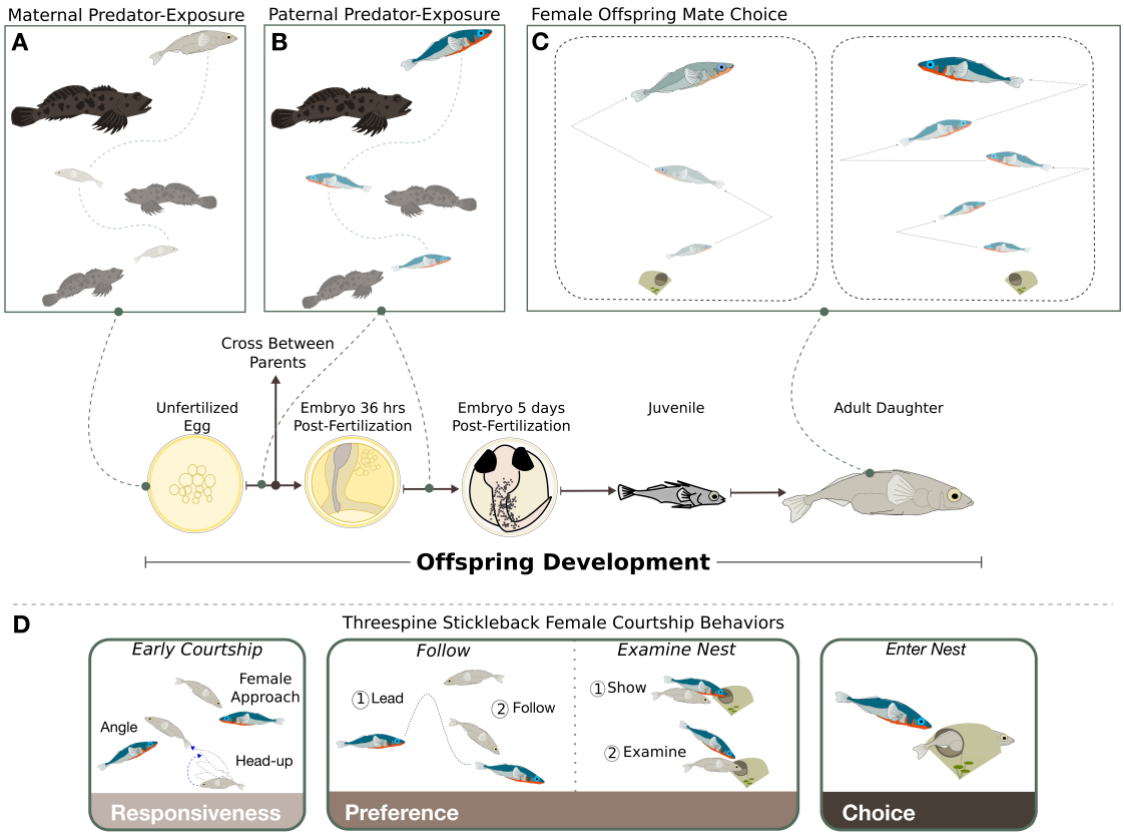


**Figure 1.1. Experimental methods through offspring development.** We exposed mothers, fathers, both, or neither to a model predatory fish at developmentally appropriate times to produce four treatments: control, predator-exposed mothers, predator-exposed fathers, predator-exposed parents. (A): To produce predator-exposed females we subjected females to the model predator for 30s each day at a random time of day during the period that females were developing a clutch of eggs. (B): We exposed fathers to the model predator twice (pre- and post-mating): a predator model was moved through their nesting tank for 30s 15 minutes before the courtship trial and for two minutes on day 3 of egg care when embryos did not have fully developed eyes. Offspring experienced no direct visual predation cues. (C): We tested the preferences and mate choices of adult daughters in standard no-choice trials with wild-caught males that varied in sexual signals (from dull blue eyes and red throat color and less vigorous courtship (left) to more colorful males who perform vigorous conspicuous courtship behavior (right)). (D): Stickleback courtship proceeds through four sequential stages. The early courtship stage indicates female interest in mating. Following to the nest is a common metric of female preference that restricts the cues assessed to those related to male phenotype (i.e. color signals and courtship behavior; (Head, Kozak, & Boughman, 2013; Head, Price, & Boughman, 2009; Kozak & Boughman, 2009). Examining the nest is also commonly used as a metric of female preference, and reflects male sexual signals and nest characteristics (Albert, 2005; Kozak, Reisland, & Boughman, 2009). Finally, entering the nest to spawn is a direct measure of female choice.

females, during egg formation (Figure 1.1A), and for males pre-mating and during egg care (Figure 1.1B). To produce predator-exposed females we subjected females to the model predator for 30s 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)). To produce predator-exposed males we subjected fathers to the model predator twice (pre- and post-mating). Each experimental male was placed in his own nesting tank (76-L, 61 cm x 30 cm x 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. For predator exposed males, we moved a predator model through their nesting tank for 30s 15 minutes before the courtship trial.

We then crossed parents under standardized 'no choice' conditions (following (Head et al., 2009; Nagel & Schluter, 1998; R. M. Tinghitella et al., 2013)). Briefly, we gently introduced the female into the male's tank through a tube with a false floor. After a two-minute acclimation in the tube, the pair was allowed up to 20 minutes to spawn. At the end of a successful cross, we returned females to holding tanks. Males remained in their nesting tanks to perform paternal care. The second predator exposure for fathers was for two minutes on day 3 of egg care (following (Stein & Bell, 2014)) when embryos did not have fully developed eyes (Swarup, 1958). Each 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 cross. Spawning success did not differ among parental predator-exposure cross combinations ($\chi^2 = 5.75$, df = 3, p = 0.12). It is possible that treated fish may have responded to disturbance associated with the predator model, and not just visual exposure to the model. In this experiment, we were interested in predation risk as a representative ecological stressor and in capturing any and all consequences of such stressors.

To assess direct effects of exposure to the predator-model on parents, we looked at differences in paternal care between predator-exposed and unexposed fathers and

differences in female courtship behavior between predator-exposed and unexposed mothers. We recorded all female behaviors during crosses and all parental care behaviors of males (including nest visits, number and duration of nesting and fanning bouts, and total time spent at the nest) for five minutes each day beginning one day post-fertilization until 16 days post-fertilization when we removed the father from the tank. Direct predator-exposure did influence parents' behavior, suggesting that our treatments were true stressors. Maternal predator-exposure reduced conspicuous early courtship behaviors of mothers by 50% relative to unexposed mothers ($F_{1,22.78}$=4.90, p=0.04; Figure 1.2A). This may be a behavioral strategy to avoid predation. Predator-exposed fathers made 20% fewer visits to the nest than unexposed fathers (4.5 ± 0.50 vs 5.3 ± 0.51 visits—means +/- S.E.; $F_{1,281}$=4.53, p=0.03; LMM, random = day of care nested within father ID; Figure 1.2B) and, when crossed with a predator-exposed mother, reduced their time spent fanning the nest by 37% ($F_{1,281}$=8.80, p=0.003; Figure 1.2C). Thus, both direct predation risk to fathers and maternal predation risk influenced paternal care.

*Measuring Daughters' Mating Behavior*

Following crosses, we raised the offspring of crosses to sexual maturity (approximately one year of age), housing them by family. Family tanks within each treatment were positioned at random within the laboratory and all were outfitted and cared for identically. We fed stickleback fry live *Artemia* nauplii and juveniles a mixture of live *Artemia* and chopped bloodworms daily. We assessed the mating behavior of female offspring from all four treatments in no-choice courtship trials with wild-caught males who were collected from the Chehalis River, WA in summer 2016 (Figure 1.1C). As before, each male was placed in his own nesting tank and allowed to build a nest.

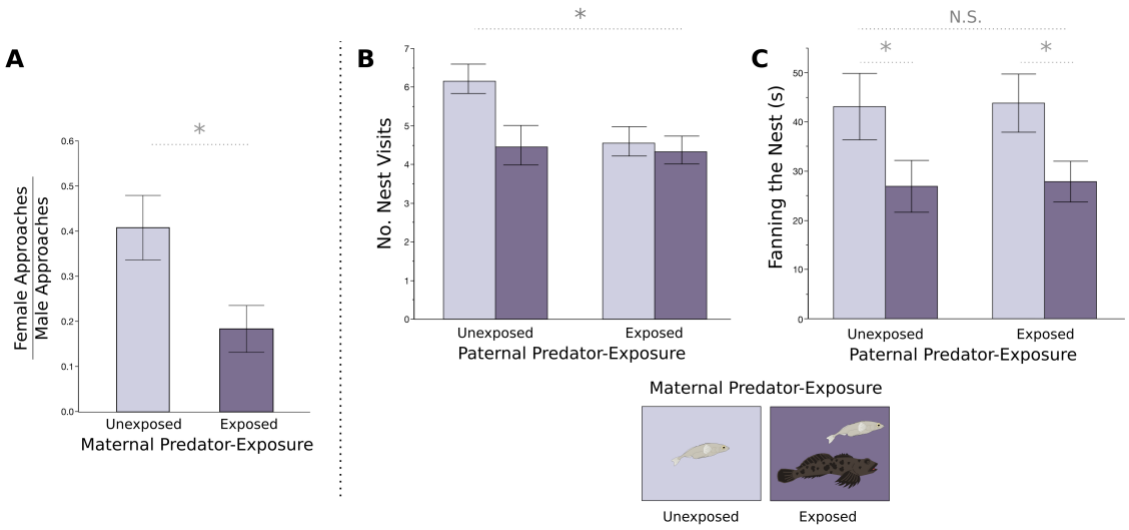


**Figure 1.2**. **Maternal mating behavior and paternal care under direct predation risk.** Exposure to the model sculpin altered the courtship behavior of exposed females and the parental care behavior of exposed males. (A): Predator-exposure reduced the mating responsiveness of mothers, which we measured as reciprocated approaches of male suitors, a conspicuous courtship behavior ($N_{Unexposed}$=14, $N_{Exposed}$=12; error bars are ± 1 S.E.). (B): Predator-exposed fathers made fewer visits to the than unexposed fathers (C): Maternal predator-exposure impacted the amount of time males spent fanning the nest, so males' parental care depended both on their own experience with the predator model and the experience of their mates. (For parental care analyses, $N_{Unexposed}$=9, $N_{Exposed}$=10; error bars are ± 1 S.E.). Together, these observations demonstrate direct effects of the model predator treatment on both parents, which may result in parental effects on offspring mate choice.

When a female became gravid, we randomly assigned her to a male with a readied nest. During courtship trials, we recorded all female and male courtship behaviors (Table S1) using the event recorder JWatcher (Blumstein, Evans, & Daniel, 2006). A trial was considered complete after 20 minutes elapsed or when the female entered the nest. If a female entered the nest of a male, we gently encouraged her to leave the nest with an aquarium net and concluded the trial to prevent spawning. Each adult daughter underwent a single no-choice trial. We used wild-caught males in up to three mate choice trials, but minimized the effects of 'male ID' on outcomes by assigning males randomly to females

from different treatments for each trial and ensuring that among males, females from different treatments were presented in random order and with different time intervals between courtship trials. Only trials in which the male tended to his nest (indicating nest-building was complete) and neither fish displayed anxiety-suggesting behaviors (e.g. nosing the aquarium wall, hiding for the duration of the trial) were included in our analyses (n=70 included, n=44 excluded trials). Daughters did not differ in age ($F_{3,14.23}$ = 3.03, p = 0.06) or size (mass/length; $F_{3,13.2}$ = 0.65, p = 0.60) at the time of their courtship trials.

The mate preferences and choices of female sticklebacks are dependent on a variety and combination of male sexual signals (Künzler & Bakker, 2001), most notably, conspicuous visual color signals (Milinski & Bakker, 1990) and courtship behaviors of males (Rowland, 1995), and body size (Head et al., 2013; Kraak, Bakker, & Mundwiler, 1999; Rowland, 1989). Females from several populations prefer males with extensive red throat coloration and blue eye coloration (Milinski & Bakker, 1990; Rowland, 1994). Male traits are also contextually plastic (Head, Fox, & Barber, 2017; Hiermes, Rick, Mehlis, & Bakker, 2016), so there can be within-male variation between trials. We quantified the red throat area and blue eye area of wild-caught males used in no-choice courtship trials from photographs taken immediately before and immediately after each trial (see Appendix). We also obtained a measure of body length in millimeters for each male (from the anterior extent of the mouth to the caudal extent of the tail).

*Statistical Analysis*

Stickleback courtship proceeds through four sequential stages, each indicating increasing levels of female interest (Kozak et al., 2009): early courtship, following, nest

examination, and nest entering (Figure 1.1D). These stages are not always modified in the same way by direct female experience (R. M. Tinghitella et al., 2013). Thus, important parental effects could be missed by analyzing all courtship stages together or choosing one to approximate the others. Our basic modeling approach was to test for parental effects on each of the four stages of courtship. More specifically, we asked whether daughters' mating behavior (indicating responsiveness, preference, and choice) depended on the interaction of parental predator exposure and her male mate's sexual signals in linear (and generalized linear) mixed models.

We first used PCA as a variable reduction technique to obtain a single measure of male sexual signals (color and behavior) for each stage of courtship (Table S2). This allowed us to assess daughters' interest in males that varied in the overall conspicuousness that is attractive to predators and to account for the sexually selected behaviors that happen at different stages of courtship. We conducted all LMM and GLMM analyses with the first principal component from these PCAs (Male Signals PC1), as it captured the most conspicuous secondary sexual traits that are attractive to both female conspecifics and predators; higher values of each Male Signals PC1 described males with greater red throat and blue eye area who performed more conspicuous zig-zag behaviors. While we assessed the mating behavior of daughters from each treatment at each of the distinct stages of courtship, a female's behavior at one stage is unlikely to be completely independent of her behavior at other stages. We accounted for potential collinearity between stages by including all female behaviors at preceding stages as a covariate in each of our models. Here, again, we used PCA, this time to generate a 'female preceding behaviors' PC for each stage of courtship (Table S2). For

instance, the PCA to generate PC1 for the 'follows' stage of courtship included only female early courtship behaviors (angle, head-up, female approach). All behaviors included in PCAs were scaled for duration of the courtship trial. Next, for each courtship stage, we first produced and visualized a preference function for each treatment using PFunc (Kilmer et al., 2017). To test for differences in preference function shape across parental effects treatments, we ran two separate LMMs (early courtship, follow, examine nest) or GLMMs (enter nest), one with linear male signal terms and a second with quadratic male signal terms to test for linear and/or quadratic female responses (Fowler-Finn & Rodríguez, 2011). The models also included female offspring treatment, preceding female behaviors and male length as covariates, and male ID and family nested within treatment as random effects. We compared the two LMMs or GLMMs using AIC to determine whether female preferences were better modeled as linear (open) or quadratic (closed) functions. In these models, a significant interaction between female offspring treatment (parental effects) and male signals on female courtship behavior indicates differences in preference function shape among treatments. When we uncovered a significant interaction, we used model parameter estimates of interaction terms to describe control-to-treatment differences in function slope (see below). For courtship stages at which there was no significant interaction between female offspring treatment and male signals on female courtship behavior (i.e. no differences in function shape among treatments), we determined whether there was a fixed effect of female offspring treatment on mate choice (i.e. preference functions differ in height but not shape). We performed all LMMs using *lmer* and all GLMMs using *glmer* in the lme4 package (Bates,

Maechler, Bolker, & Walker, 2017) and effects testing using likelihood ratio tests with *mixed* in the afex package (Singmann, Bolker, Westfall, & Aust, 2018) in R.

*Assessing Parental Effects on Sexual Selection using GAMMs*

While LMM/GLMMs inform the direction and magnitude of selection among treatments, comparisons of the shape of selection among treatments are done by informal comparison (Bailey, 2008; Fowler-Finn & Rodríguez, 2012). Here, we advocate a statistical approach with generalized additive mixed models (GAMMs) that model relationships using nonparametric smooth functions rather than assuming parametric relationships between variables (Wood 2006) and allow us to make pairwise comparisons of preference function shape between treatments. GAMMs and similar nonparametric analyses have been used previously to model natural selection (Morrissey & Sakrejda, 2013; Schluter, 1988; Schluter & Nychka, 1994) and are particularly useful when the shape of selection is unknown or more nuanced than straight lines or unimodal functions. GAMMs thus allow us to describe the shape of female preference functions without making prior assumptions about function shape and provide a key advantage over more traditional LMM/GLMM methods to assess selection (Lande and Arnold 1983) and mating preferences (Fowler-Finn and Rodriguez 2011; Rodriguez et al. 2013), particularly in study systems in which it is not feasible or recommended to test females repeatedly with different males. Because GAMMs can be overfitted and are sensitive to small changes in data (Wood, 2006), we interpret our GAMM outcomes in conjunction with our LMM/GLMM analyses, but the methods described here may also be used independent of LMMs/GLMMs when study design allows for larger sample sizes.

When the LMM/GLMM indicated differences in function shape among treatments at a given courtship stage we ran two GAMMs with nonparametric smooths (this only occurred at the Follows stage). We visually inspected our preference functions to determine GAMM smoothing parameters as described in Kilmer et al. (2017). The first GAMM contained a single smoother and thus fit a single function representing the response of females to Male Signals (PC1) across all parental effects treatments; the second contained separate smoothers for each treatment and thus fit a function to each treatment. Each full model also included female offspring treatment, preceding female behaviors (PC1) and male length (mm) as covariates, and family nested within female offspring treatment and male ID as random effects. We used AIC to compare the two GAMMs. If the GAMM with separate smoothers produced a better fit (lower AIC), this indicated that daughters' behavior in one or more treatments was best modeled with non-linear functions and that function shape differed between treatments.

When the GAMM analysis indicated that function shapes were non-linear and differed among treatments, we then made pairwise comparisons between treatment-level preference functions by creating two nested GAMM models for each pairwise comparison. In addition to our treatment-level smoothing parameters, for a given comparison, we also obtained a single smoothing parameter for the subset of the data containing individuals from the two treatments being compared and a single smoothing parameter for the two treatments not being compared using PFunc (Kilmer et al., 2017). For each pairwise comparison between treatments, the first (null) model contained a smoother for the treatments of interest combined over Male Signals (PC1) as well as a smoother for the other two treatments over Male Signals (PC1). The second model

contained separate smoothers for the two treatments of interest, each over Male Signals (PC1) as well as the single smoother for the other two treatments over Male Signals (PC1). Comparing these two models allowed us to determine if modeling behavior of daughters from the two treatments of interest with one smoother was significantly different (or not) from modeling the two treatments with separate smoothers; no difference between models indicates that the function shapes of the two treatments did not differ. The male ID random effect was nonsignificant in all of our original GAMMs (above), so to reduce model complexity for pairwise comparisons (an important consideration with relatively small datasets) we removed this effect. We constructed all GAMMs using *gam* and tested whether the separate and single smoother treatment comparison models were different using *anova.gam* in the mgcv package (Wood, 2018) in R v 3.3.1 (RStudio v 0.99.903).

## RESULTS

*Parental Effects on Mating Responsiveness, Preference, and Choice*

At the early courtship stage, female behavior was unrelated to the sexual signals of her mate (LMM interaction effect was not significant, Table 1.1A). This is not unexpected, as early courtship behaviors signify daughters' responsiveness, or willingness to mate, rather than assessment of male signals. In other words, at this stage of courtship, we found no parental effects on daughters' preference function shape, but strong effects on function elevation (Figure 1.3; $LMM_{ParentalEffects}$: $\chi^2 = 9.91$, df = 3, p = 0.02). Predator-induced parental effects led daughters whose mother, father, or both were exposed to the predator to perform 63-74% fewer early courtship behaviors than daughters of unexposed parents (Figure 1.4A; effect sizes determined using LS means).

Parental effects influenced offspring behavior in ways that depended on male sexual signals at later stages of courtship. Daughters' tendency to follow a male to the nest depended on whether parents experienced predation risk and the sexual signals of their mates and were better modeled with linear, rather than quadratic, functions ($LMM_{Parental\ Effects*Male\ Signals}$: $\chi^2 = 11.69$, df = 3, p = 0.009; Table 1.1B; Figure 1.3a-d;). Stickleback mate preferences are typically open-ended (linear, with a positive slope) for brightly colored, vigorously courting males (Boughman, 2001; Milinski & Bakker, 1990; R. M. Tinghitella et al., 2013), and control daughters preferred to follow bright, showy males, as expected (Figure 1.3a). In contrast, daughters from treatments in which only one parent was predator-exposed [predator-exposed mothers (Figure 1.3b) and predator-exposed fathers (Figure 1.3c)] had preference functions with shallower slopes compared to control, preferring less conspicuous mates than did control daughters (Table 1.2A). However, daughters of predator-exposed parents had a positively sloped preference function that did not differ from that of control daughters (Table 1.2A). Single-parent predator-predator exposure produced daughters with preferences that differed from joint parental predator-exposure, but maternal and paternal predator-exposure did not produce significant differences in daughters' preferences (Table 1.2A). Thus, maternal and paternal effects independently relaxed mate preferences of daughters. We found no evidence of parental effects on nest examination, perhaps because there is little cost to examining a nest once the female is in close physical proximity (Figure 1.3; LMM effects in Table 1.1C).

At the final stage of courtship, females decide to enter the males' nest to deposit eggs (mate choice) or abort the courtship interaction. Whether or not daughters ultimately

entered the males' nest to release eggs also depended strongly on parents' predator exposure, but the interaction between male signals and parental predator exposure on the likelihood that daughters entered the nest was only marginally significant (Table 1.1D; Figure 3e-h). There was a strong fixed effect of parental predator exposure on enters; daughters of predator-exposed parents were three times less likely to enter the nest than control daughters (Table 1.1D; Figure 1.4B). Differences between mating decisions (enters) and behavior at earlier stages of courtship may stem from the additional information females gain at later stages in courtship, which include most notably, visual and chemical cues from the nest that we did not measure.

**Table 1.1.** Describing preference functions using LMMs and GLMMs. At each courtship stage, one model fit linear functions of female behavior over male signals, and a second model fit quadratic preference functions over male signals. The AIC of the model that produced the better fit (linear/open functions vs quadratic/closed functions) is bolded in the left column of each table. All models also included family nested within treatment and male ID as random effects. (LMMs: A-C; GLMMs: D)

A. Early Courtship.

| *Linear* | | $\chi^2$ | df | P |
|---|---|---|---|---|
| | Treatment | 9.91 | 3 | **0.02** |
| AIC=**-288.46** | Male Signals PC1 | 0.05 | 1 | 0.82 |
| df=12 | Treatment*Male Signals PC1 | 0.65 | 3 | 0.89 |
| | Preceding Behaviors | - | - | - |
| | Male Length | 3.31 | 1 | 0.07 |
| *Quadratic* | | $\chi^2$ | df | P |
| | Treatment | 10.65 | 3 | 0.01 |
| AIC=-260.92 | (Male Signals PC1)$^2$ | 1.39 | 2 | 0.50 |
| df=16 | Treatment*(Male Signals PC1)$^2$ | 5.15 | 6 | 0.53 |
| | Preceding Behaviors | - | - | - |
| | Male Length | 3.80 | 1 | 0.05 |

B. Follow.

| *Linear* | | $\chi^2$ | df | P |
|---|---|---|---|---|
| | Treatment | 3.68 | 3 | 0.30 |
| AIC=**-400.24** | Male Signals PC1 | 13.39 | 1 | **0.0003** |
| df=13 | Treatment*Male Signals PC1 | 11.69 | 3 | **0.009** |
| | Preceding Behaviors | 70.47 | 1 | **<0.0001** |
| | Male Length | 1.70 | 1 | 0.19 |

| *Quadratic* | | $\chi^2$ | df | P |
|---|---|---|---|---|
| | Treatment | 5.94 | 3 | 0.11 |
| AIC=-368.15 | (Male Signals PC1)$^2$ | 14.64 | 2 | 0.0007 |
| df=17 | Treatment*(Male Signals PC1)$^2$ | 20.02 | 6 | 0.003 |
| | Preceding Behaviors | 71.28 | 1 | <0.0001 |
| | Male Length | 3.29 | 1 | 0.07 |

C. Examine Nest.

| *Linear* | | $\chi^2$ | df | P |
|---|---|---|---|---|
| | Treatment | 2.53 | 3 | 0.47 |
| AIC=**-359.29** | Male Signals PC1 | 0.07 | 1 | 0.79 |
| df=13 | Treatment*Male Signals PC1 | 4.31 | 3 | 0.23 |
| | Preceding Behaviors | 0.00 | 1 | 0.96 |
| | Male Length | 1.53 | 0.22 | 0.19 |
| *Quadratic* | | $\chi^2$ | df | P |
| | Treatment | 4.84 | 3 | 0.18 |
| AIC=-326.38 | (Male Signals PC1)$^2$ | 1.21 | 2 | 0.55 |
| df=17 | Treatment*(Male Signals PC1)$^2$ | 10.13 | 6 | 0.12 |
| | Preceding Behaviors | 0.54 | 1 | 0.46 |
| | Male Length | 1.99 | 1 | 0.16 |

D. Enter Nest.

| *Linear* | | $\chi^2$ | df | P |
|---|---|---|---|---|
| | Treatment | 5.24 | 3 | 0.16 |
| AIC=82.14 | Male Signals PC1 | 0.08 | 1 | 0.78 |
| df=12 | Treatment*Male Signals PC1 | 5.33 | 3 | 0.15 |
| | Preceding Behaviors | 13.93 | 1 | **0.0002** |
| | Male Length | 2.21 | 1 | 0.14 |
| *Quadratic* | | $\chi^2$ | df | P |
| | Treatment | 9.60 | 3 | **0.02** |
| AIC=**76.51** | (Male Signals PC1)$^2$ | 0.39 | 2 | 0.82 |
| df=16 | Treatment*(Male Signals PC1)$^2$ | 12.11 | 6 | 0.06 |
| | Preceding Behaviors | 15.36 | 1 | **<0.0001** |
| | Male Length | 3.00 | 1 | 0.08 |

*Pairwise Comparisons of Maternal, Paternal, and Joint Parental Effects using GAMMs*

At the follows stage of courtship, when stickleback researchers typically assess female preference functions, we did indeed find differences in the direction and slope of the preference function among parental effects treatments (Table 1.1B). Thus, we used GAMMs to further probe these differences without making assumptions about the shape of the functions. In support of our LMM results at this stage, the GAMM that included an

interaction term between female offspring treatment and male signals was a better fit for the data than one that did not include an interaction term ($\Delta AIC = 4.55$; Table S3). Further, our GAMM analyses probing pairwise differences between treatment functions supported the idea that the combined effects of maternal and paternal predator-exposure on daughters' preferences were not cumulative (additive or multiplicative) (Table 1.2B). Predator exposure to mothers (Figure 1.3b) and fathers (Figure 1.3c) independently shifted daughters' preferences at the follows stage in the same direction, toward less conspicuous males, while control daughters (Figure 3a) and those of parents who were both exposed to the model predator preferred brightly colored males that courted vigorously (Figure 3d). Further, GAMM smoother effects, which indicate whether preference function shape is linear or non-linear (Table S3B) show that control and predator-exposed parents daughters have open, linear preference functions, while daughters of predator-exposed mothers and fathers had closed but non-linear preference functions. Again, we interpret our GAMM pairwise comparison results with some caution, given that GAMMs are sensitive to smaller datasets, but note that the GAMM outcomes are in complete agreement with the LMM outcomes and additionally inform us that some functions are linear while others are not. We encourage the use of this and similar analyses that allow for more flexible modeling of the shapes of preference functions and other function valued traits.

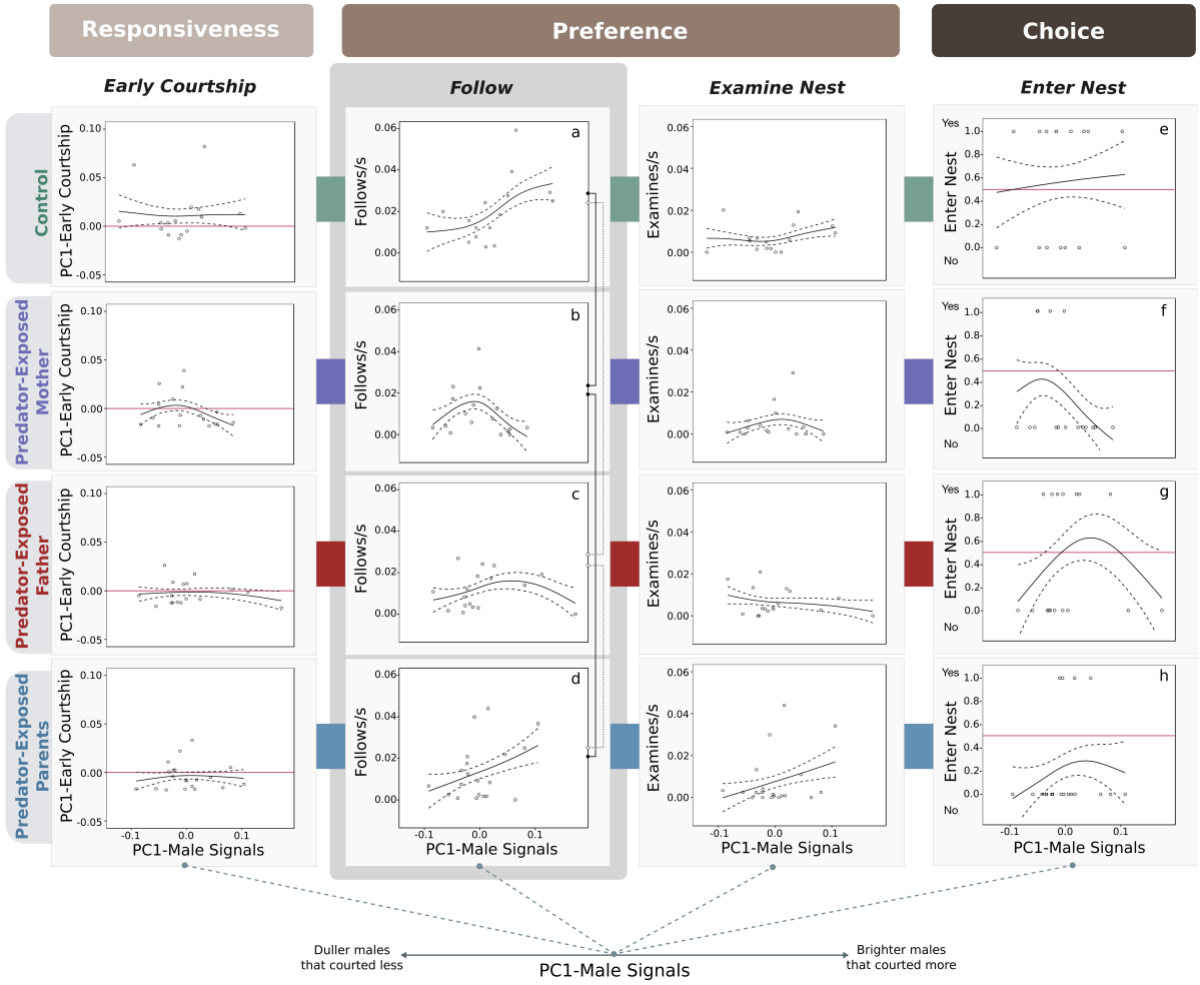


**Figure 1.3. Maternal and paternal effects independently change the direction of sexual selection and are not cumulative.** We constructed treatment-level functions (non-parametric smooths and their standard errors) at each of four stages of courtship. Each open circle represents the behavior of one daughter. The x-axis is a metric of sexually selected male traits (PC1 from a PCA combining male throat color, eye color, and courtship behaviors; Table S2): duller males, fewer zig-zags to the left and brighter males, more zig-zags to the right. The y-axis shows the behavior(s) performed by daughters at each courtship stage. The graphs for Early Courtship contain a red reference line at y = 0 and graphs for Enter Nest at y=0.5 to aid visual differentiation of function heights. We found evidence of differences in function direction/magnitude and shape across treatments at the Follows stage of courtship using LMMs and GAMMs, respectively (courtship stage surrounded by large grey rectangle). Brackets connecting treatments indicate significantly different function direction and shape (see Table 1.2).

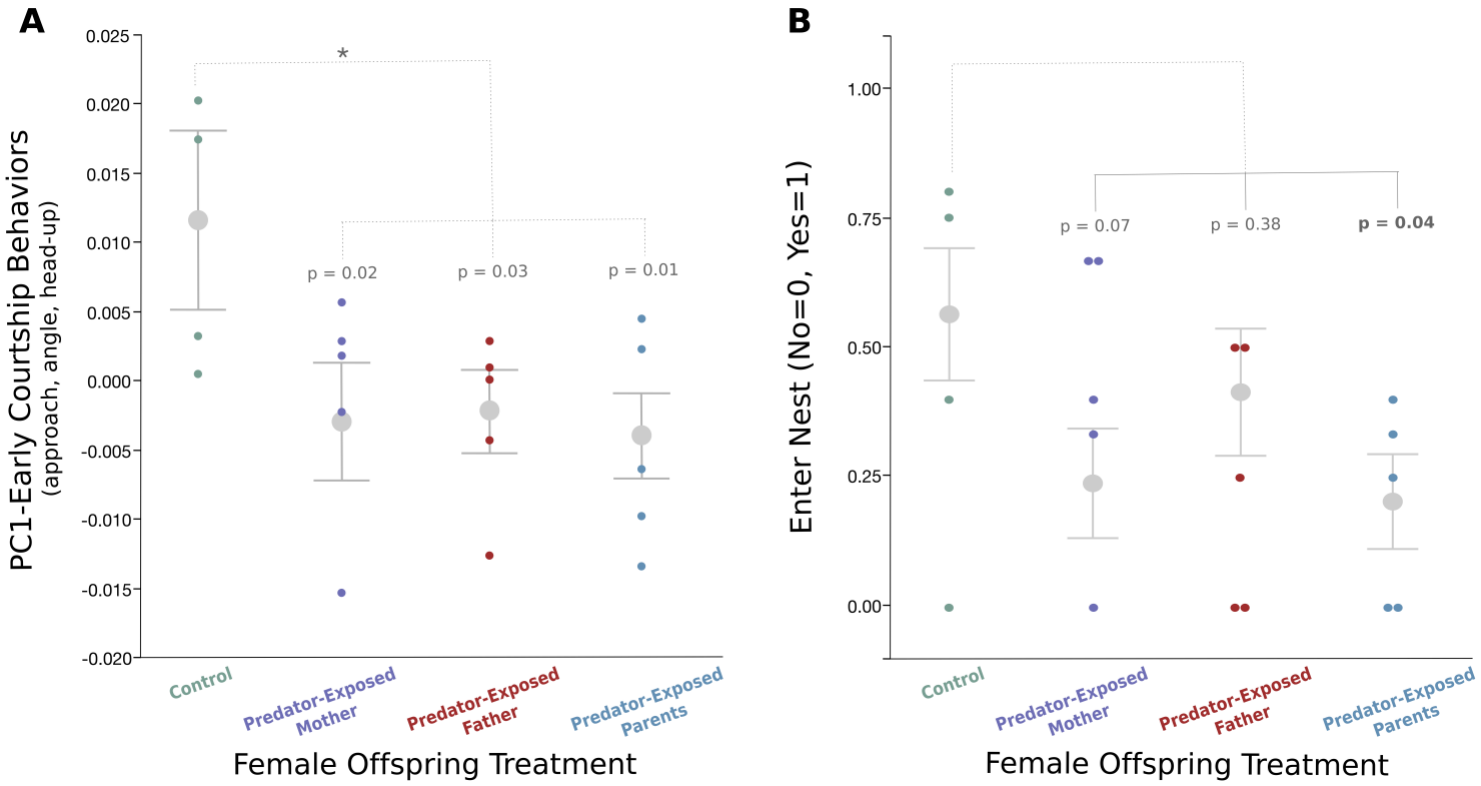


**Figure 1.4. Parental effects on mating responsiveness (early courtship) and mate choice (entering the nest).** (A): Daughters of predator-exposed mothers, fathers, and parents perform fewer conspicuous early courtship behaviors than control daughters. Control-to-treatment comparisons using Dunnett's test. Grey dots and bars indicate treatment estimates ± S.E.: predator-exposed mother (-0.02 ± 0.01, z = -2.63), predator-exposed father (-0.02 ± 0.01, z = -2.51), and predator-exposed parents (-0.02 ± 0.01 , z = -2.91). Smaller, colored dots within a treatment indicate family means. LS Means ± S.E.: control (0.012 ± 0.005), predator-exposed mother (-0.003 ± 0.004), predator-exposed father (-0.003 ± 0.004), predator-exposed parents (-0.004 ± 0.004). (B): Daughters of predator-exposed parents are less likely to enter the nest than control daughters. Control-to-treatment comparisons using Dunnett's test: predator-exposed mother (-2.94 ± 1.36, z = -2.156), predator-exposed father (-1.51 ± 1.64, z = -1.30), and predator-exposed parents (-3.67 ± 1.50, z = -2.44). LS Means ± S.E.: control (1.30 ± 0.91), predator-exposed mother (-1.64 ± 0.94), predator-exposed father (-0.21 ± 0.77), predator-exposed parents (-2.37 ± 1.06).

**Table 1.2.** Pairwise treatment comparisons of preference function direction, magnitude, and shape for the follows stage of courtship.

A. Differences in preference function direction/magnitude using LMMs and parameter estimates.

| | *Predator-Exposed Mother* | *Predator-Exposed Father* | *Predator-Exposed Parents* |
|---|---|---|---|
| *Control* | -0.11 ± 0.05, df = 59.86, t = -2.43, **p = 0.02** | -0.09 ± 0.04, df = 56.66, t = -2.29, **p = 0.03** | -0.01 ± 0.04, df = 58.26, t = 0.18, p = 0.86 |
| *Predator-Exposed Mother* | - | 0.02 ± 0.04, df = 58.90, t = 0.54, p = 0.59 | -0.12 ± 0.05, df = 59.80, t = -2.48, **p = 0.02** |
| *Predator-Exposed Father* | - | - | -0.10 ± 0.04, df = 57.12, t = -2.30, **p = 0.03** |

B. Differences in preference function shape using GAMMs.

| | *Predator-Exposed Mother* | *Predator-Exposed Father* | *Predator-Exposed Parents* |
|---|---|---|---|
| *Control* | $F_{7.28,46.75}$=2.47, **p=0.03** | $F_{1.57,43.92}$=10.12, **p<0.001** | $F_{9.55,46.29}$=1.73, p=0.10 |
| *Predator-Exposed Mother* | - | $F_{0.72,\ 51.31}$=2.19, p=0.15 | $F_{3.85,45.95}$=3.21, **p=0.02** |
| *Predator-Exposed Father* | - | - | $F_{2.67,50.82}$=3.43, **p=0.03** |

## DISCUSSION

Predator-induced parental effects clearly extend through to sexual maturity to alter daughters' mating behavior. Single parent and joint parental predator exposure reduced daughters' mating responsiveness (early courtship stage; Figure 1.4A), maternal and paternal effects independently relaxed or reversed the direction of typical mating preferences (follow stage; Figure 1.3), and daughters whose parents both experienced predator-risk were less likely to mate at all (enters stage; Figure 1.4B). Further, control and predator-exposed parent daughters had open, linear function shape (Figure 1.3a,d), which differed from daughters of predator-exposed mothers and fathers that produced with closed, nonlinear function shapes (Figure 1.3a,b; determined using GAMMs). Taken

together, our results demonstrate that ecological experiences of parents (in this case predator exposure) impacts multiple facets of sexual selection.

We first found that environmental stress parents experienced reduced the early courtship behaviors of offspring via parental effects. Reducing conspicuous early courtship behavior could enhance the survival of daughters, increasing daughters' fitness in predator-rich environments. Here, then, within- and across-generation effects of parental predation risk on daughters' interest in mating responses are concordant, as theory predicts (Figure 1.2A and 4A; (Mousseau & Fox, 1998); but see (Walsh et al., 2015)), with parental effects decreasing daughters' conspicuous courtship behaviors. When directly exposed to ecological stressors like predation, males often develop less conspicuous ornaments and courtship behaviors (Candolin, 1997, 1998; Magnhagen, 1991), and females often choose to mate with less-conspicuous, less-preferred males ((Endler, 1983; Hedrick & Dill, 1993; Wong & Rosenthal, 2006); but see (Kim, Christy, Dennenmoser, & Choe, 2009)). These effects can be both plastic and evolutionary, providing females with direct (material) or indirect (genetic) fitness benefits (Andersson, 1994). Females gain direct benefits by associating with less conspicuous males that are less likely to draw the attention of predators to her and their offspring, and may gain indirect benefits if male offspring inherit their father's duller display and daughters inherit their mother's preference for less conspicuous traits (Bakker, 1993).

Experience-mediated changes in preference functions can dramatically alter the course of sexual selection (Chaine & Lyon, 2008; Fowler-Finn & Rodríguez, 2012). Here, we find that an ecological stressor on parents spans a generation to change sexual selection exerted by daughters. Such intergenerational effects on sexual selection offer an

additional explanation for the maintenance of genetic variation in sexually selected signals and behaviors (Kirkpatrick & Ryan, 1991). What explains the non-cumulative effects of maternal and paternal predator-exposure on daughters' mating preferences? It is possible that daughters of predator-exposed parents showed a 'recovery' of preferences for brighter males (particularly prominent at the follows stage) due to social buffering (i.e. when social interactions like parental care mitigate the costs of stressors; (Beery & Kaufer, 2015; Faustino, Tacão-Monteiro, & Oliveira, 2017)). Stickleback males can assess the experience their mates have had with predators, and decrease their courtship behavior (Dellinger, Zhang, Bell, & Hellmann, 2018) and parental care (McGhee, Feng, Leasure, & Bell, 2015) in response to predator-exposed females. Here, rather than finding evidence that fathers compensate for mothers' predator-exposure by increasing parental care, we similarly found that fathers exposed to the predator model reduced their number of nest visits (Figure 1.2B), and, when mated with a predator-exposed mother, reduced their time spent fanning the nest (Figure 1.2C). Therefore, changes in paternal care in response to mating with predator-exposed mothers may have indirectly contributed to the maternal effects on daughters' mating behavior measured here. If social buffering is at play, fathers may compensate for maternal predator-exposure in ways we did not capture with measured parental care behaviors. For instance, fathers often chase and retrieve their free-swimming fry, behaviors thought to impart antipredator behavior to offspring (Tulley & Huntingford, 1987). That female predator-exposure influences the courtship and parental care of males indirectly suggests that female predator-exposure may affect their attractiveness. If indirect predator-exposure, via parental effects, on daughters' attractiveness works in parallel, then predator-induced parental effects could impact male

courtship and parental care via daughters' attractiveness, producing potential within- and across-population variation in reproduction and offspring developmental and rearing environments.

Alternatively, the predator risk allocation hypothesis may explain the non-cumulative effects of maternal and paternal predator-exposure (Lima & Bednekoff, 1998). The predator risk allocation hypothesis predicts that in environments where predation risk is chronically high, animals will often allocate little to predator avoidance in order to adequately forage (in this case: to obtain matings; (Ferrari, Sih, & Chivers, 2009; Lima, 1998; Lima & Bednekoff, 1998)). While effects of parental predator exposure on daughters' mating behavior do not appear to be cumulative, the perceived level of stress (stemming from the combined experience of mothers and fathers) may still be cumulative. For instance, maternal and paternal effects on daughters' mating preferences do not appear to be cumulative (Figure 1.3, follows stage), but their mating responsiveness and mating choices are reduced under joint parental effects (Figure 1.4A,B). Further, under direct predation risk, females sometimes respond in the direction opposite of expectation, showing preferences for more conspicuous males. This finding is consistent with the predator risk allocation hypothesis when direct benefits of mating with more conspicuous males are especially high (e.g. (Kim et al., 2009)). In sticklebacks, redder males are better able to defend territories (Bakker & Sevenster, 1983; R.M. Tinghitella, Lehto, & Lierheimer, 2018) and gain access to more concealed nesting sites (Kraak, Bakker, & Hočevar, 2000). Additionally, redder fathers confer an immunity advantage to offspring (Barber, Arnott, Braithwaite, Andrew, & Huntingford, 2001; Folstad, Hope, Karter, & Skorping, 1994). Taken altogether, daughters of predator-

exposed parents, who received information via parental effects suggesting they were living in a high-predation environment, may maximize their direct and indirect benefits by mating with the more conspicuous, but often higher quality, males (Andersson, 1994; Møller & Jennions, 2001), but at a lower rate.

The similarity in daughters' preference function shapes in the control and predator-exposed parents treatments may stem from interactions between maternal and paternal epigenetic changes (e.g. DNA methylation; (Champagne, 2016; Shea, Pen, & Uller, 2011)). In many systems, mothers under predation risk change hormone deposits in eggs (e.g. glucocorticoids; (Giesing et al., 2011; Love, MCGowan, & Sheriff, 2013) and the caring parent(s) often changes their parental care in the presence of predators (Ghalambor et al., 2013; Huang & Wang, 2009; Smith & Wootton, 1995; Stein & Bell, 2012). Investigating the proximate, physiological and molecular bases underlying maternal and paternal effects would provide a fuller understanding of their combined evolutionary effects on daughters' mating behavior (Badyaev & Uller, 2009).

A longstanding question in evolutionary biology is how plasticity and adaptive evolution interact to potentiate population responses to environmental change (Ghalambor et al., 2015; Pfennig et al., 2010; Walsh et al., 2016). The extent to which parental effects on offspring reproduction are adaptive depends on the degree to which parent environments are reflective of offspring environments (match or mismatch; (Burgess & Marshall, 2014; Sheriff & Love, 2013)). Recent work, however, highlights the sometimes maladaptive or insufficient nature of plastic responses in response to environmental change (Uller, Nakagawa, & English, 2013; van Baaren & Candolin, 2018), so adaptive parental effects are not a given. Here, we found that predator-induced

maternal and paternal effects independently shifted offspring preferences in the same direction, favoring duller males that courted less vigorously and reducing overall mating rates when both parents were predator-exposed, altering the course of sexual selection. Thus, when *both* parents make substantial but distinct contributions to offspring development, the experience of mothers *and* fathers can impact offspring traits, like mating, that are expressed late in life. Our findings underscore the importance of 1) characterizing the impacts of maternal and paternal effects separately and in combination and 2) examining parental effects on reproductive traits that dictate genetic contributions to the next generation.

# Chapter Two

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

(Chapter Two is published in *Evolutionary Ecology Research*, Volume 20, pp. 1-12.)

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

**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).

# 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 & Uller, 2009; Crean & 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, Krebs, & Boonstra, 2010; Sheriff & Love, 2013), in some cases resulting in adaptive offspring responses that parallel the effects of direct exposure to the same environmental stressor (Burgess & Marshall, 2014; Mousseau & Fox, 1998; Storm & Lima, 2010).

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 (Bonier, Martin, Moore, & Wingfield, 2009; Sapolsky, Romero, & Munck, 2000). The hypothalamus–pituitary–adrenal (or inter-renal) 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 (Love et al., 2013; Sapolsky et al., 2000). 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 behavioral response) through negative feedback when the glucocorticoids bind to glucocorticoid receptors and mineralocorticoid receptors in the hippocampus (Liu et al., 1997; Matthews, 2002; Sapolsky et al., 2000). Elevated glucocorticoid exposure during development is thought to decrease the number of glucocorticoid receptors and mineralocorticoid receptors (Liu et al., 1997; Love et al., 2013; Sapolsky et al., 2000); 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 & Geslin, 2008). Elevated glucocorticoids during development have effects on many offspring traits, including decreased activity and increased anxiety in zebrafish (Best, Kurrasch, & Vijayan, 2017) and slowed growth and higher corticosterone in Japanese quail (Hayward & Wingfield, 2004).

Variation in parental care also impacts development of the HPA axis (Francis & Meaney, 1999; Liu et al., 1997). 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 behaviors; thus, maternal care and stress responses depend on non-genomic maternal effects (Francis, Diorio, Liu, & Meaney, 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 behaviors than offspring that receive paternal care (McGhee & Bell, 2014). In organisms with biparental care, the removal of one parent also seems to impact stress-related hormones and behaviors; for example, zebra finches reared without a mother display higher concentrations of corticosterone relative to those reared by both parents (Banerjee & Aterberry, 2012), and California mice have both decreased survival and increased stress-related behaviors when deprived of paternal care (Glasper, Hyer, & Hunter, 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, Morgan, Bronson, Revello, & Bale, 2013) and survival (Crean, Dwyer, & Marshall, 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; Love et al., 2013; Monclús, Tiulim, & Blumstein, 2011; Sopinka, Capelle, Semeniuk, & Love, 2016) and the care parents provide to offspring (Ghalambor et al., 2013; Magnhagen, 1992; Stein & Bell, 2012; Vitousek, Jenkins, & Safran, 2014). There

are numerous examples of predator-induced parental effects on offspring morphology (Agrawal, Laforsch, & Tollrian, 1999; Stein & Bell, 2014), anti-predator behavior (Storm & Lima, 2010), learning (Roche et al., 2012), life history (Walsh et al., 2015), and reproduction (Lehto & Tinghitella, in revision).

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 behavior, 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 behavior 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 defense) and fry (chasing and retrieving fry that stray from the nest and continued territory and offspring defense) for 3–15 days (Wootton, 1984; Tulley and Huntingford, 1987(Tulley & Huntingford, 1987; Wootton, 1984). Paternal care behavior is also modified (decreased) by direct exposure to predators (Stein & 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 & 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 behavior) 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' behavior (Lehto & Tinghitella, in revision). In that study, joint parental effects impacted the mating behavior 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, colorful 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 & 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 (Head et al., 2009; Nagel & Schluter, 1998; R. M. 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 & 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 & Tinghitella, in revision). 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 behavior in no-choice mating trials for a study of parental effects on mate preferences and mate choice (Lehto & Tinghitella, in revision). 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 millimeter 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 might 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 (Heinimaa & Heinimaa, 2004; Morita & Takashima, 1998; Wootton, 1973), 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 2.1; Figure 2.1). 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, $z = 2.84$, $P = 0.02$; Figure 2.1; 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 2.1). Female length was not a significant covariate on egg cortisol ($P = 0.35$) or egg size ($P = 0.36$).

**Table 2.1.** 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.

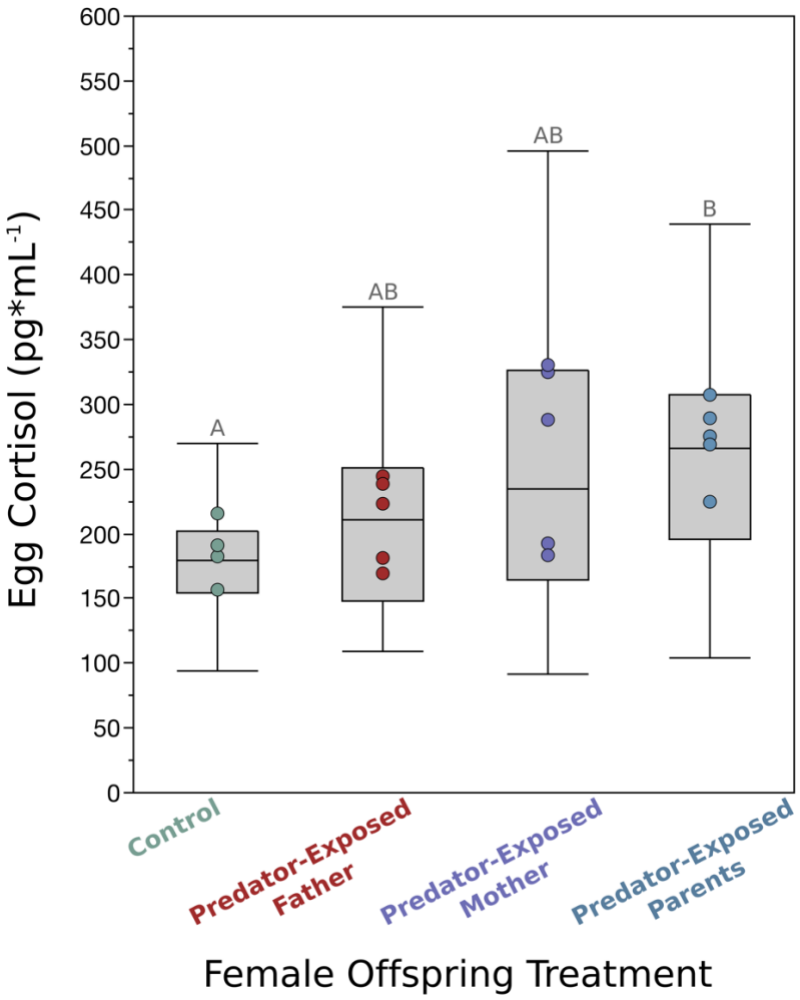


**Figure 2.1. Predator-induced parental effects increase the cortisol concentration in daughters' eggs.** Boxplots show the egg cortisol of daughters when neither parent (control), their mother (predator-exposed mother), their father (predator-exposed father), or both parents (predator-exposed parents) 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 (Best et al., 2017; Hayward & Wingfield, 2004). 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 behavioral differences consistent with adaptive stress responses in the same daughters used in this study (Lehto & Tinghitella, in revision), suggesting underlying differences in physiology. It is also possible that daughters' egg cortisol was established, perhaps epigenetically (Ho & 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 behavior (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 & Tinghitella, in revision), 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 & Tinghitella, in revision); thus it is only when both developmental exposure to cortisol (Giesing et al., 2011) *and* paternal care (Stein & 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, Catriona, & Phillip, 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 (Chirgwin, Marshall, Sgró, & Monro, 2018; Ghalambor et al., 2015). 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 behavioral 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.

# Chapter Three

## Do mom and dad know best when stressed? Predator-induced maternal and paternal effects on offspring gene expression are similar and cumulative

*Keywords*: parental effect, maternal effect, paternal effect, gene expression, predation, stress, stickleback

**ABSTRACT**

Parental experience can alter the developmental and rearing environments of offspring, resulting in parental effects on offspring traits. Which parent is the source of parental effects (mother, father, or both) can impact which traits are influenced and to what extent. Whether or not parental effects prepare offspring for their parents' environment (in an adaptive way) likely depends on the extent to which parents and offspring have similar experiences and environments. We previously showed that predator-induced maternal, paternal, and joint parental effects have different and dramatic, intergenerational impacts on the behavior and physiology of threespine stickleback offspring, suggesting that maternal and paternal effects may be underlain by different epigenetic mechanisms. Here, we ask 1) how does gene expression vary with maternal, paternal, and joint parental predator-exposure, and 2) how does gene expression

vary with parental predator-exposure when parent and offspring environments match or mismatch? We exposed threespine stickleback females and males to a predator model in a fully factorial design to produce offspring of four parental effects (indirect predator cues) treatments, where neither parent, the mother only, the father only, or both parents were predator-exposed. Then, using a split-clutch design, we exposed one half of the offspring from each family to the predator model directly, allowing us to compare offspring gene expression among sources of indirect predator cues (maternal, paternal, and joint) as well as all combinations of indirect and indirect plus direct exposure. Offspring gene expression varied with the source of parental effects: maternal and paternal effects on offspring gene expression were similar to each other, but each was different from joint parental effects. There were no differences in offspring gene expression when parent and offspring matched and mismatched, perhaps because of the animals' age at direct exposure and the specific method of predator-exposure used in this study. Maternal and paternal effects appear to be underlain by different epigenetic changes that yield independent, but perhaps additive, variation to offspring gene expression that could have an array of impacts on offspring phenotypes.

## INTRODUCTION

Parents indirectly impact traits of their offspring through parental effects, allowing near immediate intergenerational responses to environmental conditions. When environmental conditions are relatively stable, parental effects can be adaptive or preparatory in nature because the environment that a parent experiences is likely to be predictive of the environment their offspring will inhabit (Burton & Metcalfe, 2014; Mousseau & Fox, 1998; Sheriff & Love, 2013). Ultimately, parental effects allow offspring to respond not only to the environment they experience but also the environment their parents experienced (Marshall & Uller, 2007; Uller, 2008). However, the extent to which parental effects are preparatory likely depends on the agreement between parental and offspring environment (Burgess & Marshall, 2014; Uller et al., 2013); when there is a mismatch in parental and offspring environment, traits that might otherwise prepare offspring for parental environments can instead be detrimental. Rampant environmental change increases the potential for environmental mismatch and likely reduces the extent to which parents and offspring have similar experiences (both experiencing high temperatures or drought or low population densities, for instance).

Direct environmental experience and parental effects (indirect environmental cues provided to offspring) are often assumed to induce parallel changes in phenotypes (Moore, Wolf, & Brodie III, 1998; West-Eberhard, 2003). Yet direct experience and indirect cues from the same stressor may instead induce changes in different molecular pathways (i.e. affecting different genes and/or evoking different epigenetic mechanisms (e.g. DNA methylation, histone modifications, non-coding RNAs)) . We might then expect an organism receiving both direct and indirect cues about the same environmental

condition to initiate a more dramatic response (to be additive) than one receiving only direct *or* indirect cues (cue-integration theory, (Dall, McNamara, & Leimar, 2015; Leimar & McNamara, 2015)). However, recent evidence suggests that gene expression profiles following from personal experience with predation risk and paternal cues about predation risk are not additive, but instead redundant (Stein, Bukhari, & Bell, 2018). Offspring in that study showed the same phenotypic and molecular responses to their own and their father's exposure to predators. If direct exposure and indirect cues change similar molecular pathways and work in a threshold-like fashion or if indirect cues parents provide are reliable indicators of predation risk in the offspring environment, then indirect cues may be sufficient to elicit offspring responses to stressors like predation.

The integration of direct and indirect information about predation risk that offspring receive may depend on which parent (or both) is the source of parental effects. Predator-induced maternal effects influence a variety of offspring phenotypes (e.g. anti-predator behavior (Giesing et al., 2011; Storm & Lima, 2010), learning (Roche et al., 2012), and life history (Walsh et al., 2015)). Maternal effects are more often studied than paternal effects, but paternal effects may be particularly important for species in which parental care is shared (e.g. many birds) or taken on solely by males (e.g. many fish) (Balshine, 2012). The role of paternal effects in shaping offspring phenotypes has been more recently appreciated (e.g. body shape (Stein & Bell, 2014), anxiety-related phenotypes (Dietz et al., 2011; McGhee & Bell, 2014), and cognitive development (Bredy, Lee, Meaney, & Brown, 2004)), and some evidence even suggests paternal effects may produce stronger offspring responses than maternal effects (Guillaume, Monro, & Marshall, 2016). Like direct and indirect cues, maternal and paternal effects

may act on different pathways, inducing different phenotypic responses to the same environmental variable or similar phenotypic responses through different mechanisms.

We found previously that maternal predator-exposure and paternal predator-exposure influenced daughters' mating behavior in similar ways in threespine stickleback (*Gasterosteus aculeatus*), reducing daughters' mating responsiveness and relaxing daughters' preferences for typically preferred bright, conspicuous males (Lehto & Tinghitella, in revision). The impacts of joint parental predator-exposure on daughters' mating behavior, however, worked in the opposite direction, with daughters of joint parental-predator exposure retaining preferences for conspicuous males. In another study, we found that joint predator-exposure elevated daughters' egg cortisol (a stress hormone; (Lehto & Tinghitella, 2019)). Taken together, these findings suggest that maternal and paternal effects may operate independently but not additively in this system. Given that maternal, paternal, and joint parental effects each seems to dramatically and quickly alter offspring characteristics, but in different ways, they may be underlain by different epigenetic mechanisms (Curley, Mashoodh, & Champagne, 2011; Heard & Martienssen, 2014; Rodgers et al., 2013; Yehuda et al., 2014), producing substantial variation in offspring phenotypes when parental experience and contributions to offspring development vary. By examining maternal, paternal, and joint parental effects on offspring gene expression, including when parent and offspring predation environments match or mismatch (Hoyle & Ezard, 2012), we can probe the mechanisms by which direct experience and indirect cues from different parents change offspring characteristics, and perhaps influence population level evolutionary trajectories, in rapidly changing environments (Dall et al., 2015; Uller, English, & Pen, 2015).

We ask, first, whether maternal, paternal, and joint parental effects impact offspring gene expression differently, given our previous work that showed that single-parent parental effects and joint parental effects operate differently on offspring behavior (Lehto & Tinghitella, in revision) and physiology (Lehto & Tinghitella, 2019). Second, we ask whether gene expression varies when parent and offspring environments match and mismatch. That is, do offspring 'prepared' for predation risk via indirect cues (parental effects) differ in gene expression when their environment is predator-free (environment mismatch) versus when they are faced with predation themselves (environment match)? Stickleback females lay energetically expensive eggs in nests that are built and defended by males who then take on all egg care and fry-guarding (van Iersel, 1953). Both parents make substantial contributions to offspring development at different timepoints, allowing us to dissect the manner in which maternal and paternal effects interact to influence offspring gene expression. We exposed adult threespine stickleback to a model predator and then crossed them to produce four parental predator-exposure treatments: no parental predator-exposure, maternal predator-exposure, paternal predator-exposure, and joint parental predator-exposure. We then exposed half of the offspring from each family directly to the predator model in a split clutch design (Figure 3.1A) to test the hypotheses that gene expression may vary 1) with maternal, paternal, and joint parental predator-exposure and 2) when parent and offspring environments match and mismatch.

## MATERIALS AND METHODS

In summer 2015 we collected adult male and female stickleback from the Chehalis River in SW Washington, USA using minnow traps and returned them to the lab at the University of Denver where we crossed them to produce four predator-exposure parental effects treatments: no parental predator-exposure, maternal predator-exposure, paternal predator-exposure, and joint parental predator exposure. In the lab, all fish were maintained inside of 110-L tanks in a temperature and light controlled room set to 17$^{o}$C and a 12:12 light:dark schedule. Adult sticklebacks were fed a diet of bloodworms and *Artemia* daily (scaled for the number of individuals per tank approaching *ad libitum*). All tanks contained a halved ceramic pot for shelter, a mesh bag filled with crushed coral, and a plastic plant. We induced maternal, paternal, or joint parental effects by exposing males and females to a model predator at developmentally appropriate times (methods detailed in (Lehto & Tinghitella, in revision)). To produce predator-exposed mothers, we swam a sculpin fishing lure (mimicking the shorthead sculpin (*Cottus confuses*), a predator of adult and juvenile stickleback in SW Washington) through the females' tank for 30s once a day at a random time of day, to reduce habituation, during egg development (following (Giesing et al., 2011; McGhee et al., 2012; Roche et al., 2012)). To produce predator-exposed fathers, we swam the same sculpin model through each nesting male's tank two times. The first exposure took place 15 minutes prior to his cross for 30s and the second was on the third day of egg care for 2 minutes, before embryos have fully developed eyes (Swarup, 1958) to eliminate visual predator cues to offspring (following (Stein & Bell, 2014)). We produced offspring of each parental effects treatment type by offering females (predator-exposed or not) the opportunity to spawn

with a randomly assigned male (predator-exposed or not) who had a readied nest inside of a 76 or 110-L tank.

Following crosses, we reared the offspring to sexual maturity (approximately one year of age). Family tanks within each treatment were positioned at random within the laboratory. Stickleback fry were fed live *Artemia* nauplii and juveniles were fed a mixture of live *Artemia* and prepared bloodworms daily. In summer 2016, we permanently removed a subset of female offspring from each family for mate choice and egg hormone testing (Lehto & Tinghitella, 2019, in revision). Approximately 16 months later, we randomly assigned the remaining adult offspring (males and females) from these crosses to be directly predator exposed or not, producing groups of fish that had one of eight different experiences: no direct or indirect (parental) exposure and direct exposure only (controls), maternal, paternal, and joint parental predator-exposure only, maternal plus direct exposure, paternal plus direct exposure, and joint parental plus direct exposure (Figure 3.1A). In half of these eight groups the parental predation environment matches the offspring environment and in the other half there is an environmental mismatch, allowing us to compare offspring gene expression among sources of indirect predator cues (maternal, paternal, and joint) as well as all combinations of indirect and indirect plus direct exposure (Figure 3.1A). We housed each split family at equal densities and sex ratios in their own 76-L or 110-L tanks. To directly expose individuals, we swam the sculpin model through the tanks of directly exposed offspring for 30s, once daily for at least 14 days. We exposed the offspring to the predator model at a random time each day.

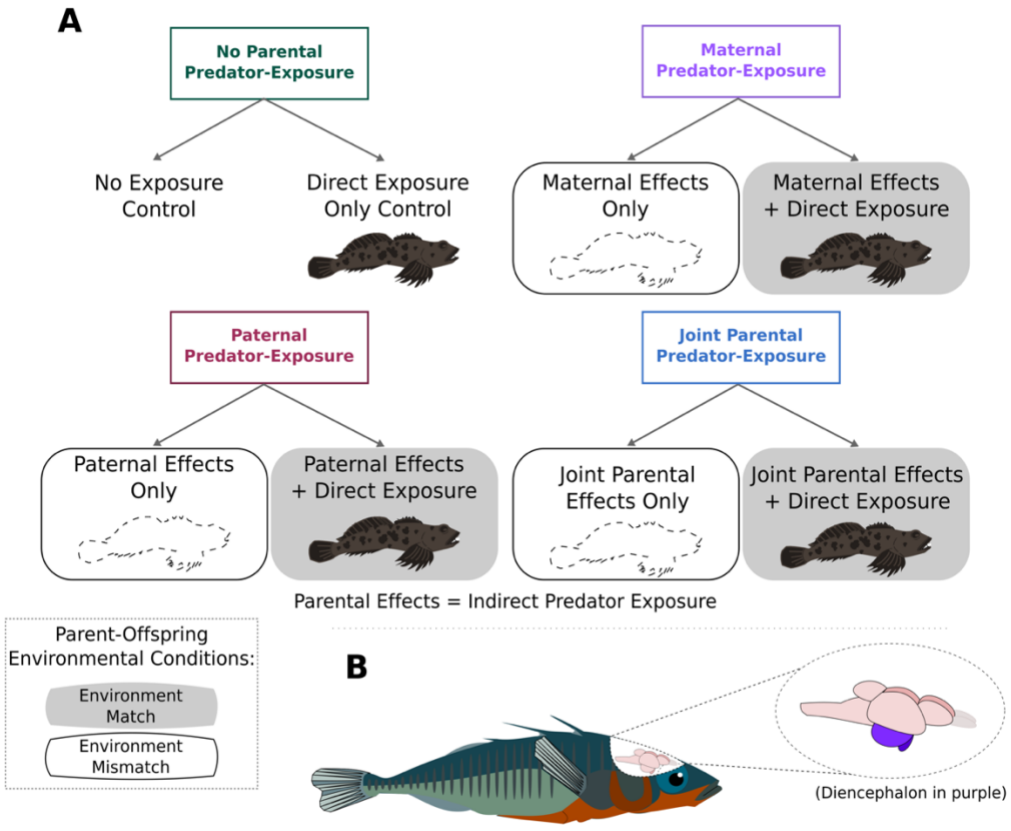


**Figure 3.1. Experimental design.** (A) Our split clutch design allowed for comparisons of gene expression patterns among maternal, paternal, and joint parental effects and when parent and offspring environments matched and mismatched. (B) The diencephalon of offspring was dissected and used for RNA-seq. The diencephalon contains structures of the HPA/I stress axis: the hypothalamus and pituitary gland, and gene expression in the diencephalon is associated with social challenges in stickleback.

We then randomly selected one fish at a time from one of the eight treatments (approximately 18-24 hours after their last direct predator-exposure), decapitated the fish, and immediately submerged the head in liquid nitrogen. Once frozen, we made an opening in the top of the skull using dissection scissors and stored the whole head in RNAlater™ in a -20°C freezer. We dissected the whole diencephalon from each brain and extracted RNA using a RNAeasy Mini Kit (Qiagen). We chose to quantify gene expression in the diencephalon because it contains the hypothalamus and pituitary gland which are structures of the hypothalamus-pituitary-adrenal (interrenal) stress axis (HPA

axis) (the endocrine axis responsible for the secretion of the glucocorticoid hormones important in vertebrate metabolism and stress responses (Bonier et al., 2009; Sapolsky et al., 2000)). Variation in the developmental environment that offspring experience like, egg hormones (especially glucocorticoids) and parental care, can lead to modifications of the HPA axis, resulting in variation in the negative feedback mitigation of glucocorticoids and offspring stress responses (Liu et al., 1997; Love et al., 2013; Matthews, 2002). In threespine stickleback, there is known variation in gene expression in the diencephalon when individuals experience social challenges (Bukhari et al., 2017; Sanogo, Band, Blatti, Sinha, & Bell, 2012), including differential expression of genes involved in hormone signaling and immune response (Bukhari et al., 2017; Greenwood & Peichel, 2015). Samples sizes for RNA-seq library preparation were N=5 fish per treatment (N = 40 total fish) spread among 2-4 families per treatment (no predator exposure and direct exposure only (controls) = 2 families; maternal effects only and maternal effects + direct exposure, N= 4 families; paternal effects only and paternal effects + direct exposure, N = 3 families; joint parental effects only and joint parental effects + direct exposure, N = 3 families; Figure 3.1A).

*Library preparation, Transcriptome sequencing, and Informatics*

Library preparation, transcriptome sequencing, and read processing and alignment were performed at Novogene Corporation using their standard methods, and described here. Novogene Corp. first evaluated RNA degradation and contamination on 1% agarose gels and checked RNA purity using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). We quantified RNA and assessed integrity using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

A total of 1 μg RNA per sample was used as input material for the RNA library preparations. They generated sequencing libraries using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and added index codes to attribute sequences to each sample. To select cDNA fragments of ~150-200 bp in length, they purified library fragments with the AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was added to size-selected, adapter-ligated cDNA and incubated at 37 °C for 15 min followed by 5 min at 95 °C before PCR (Uracil excision). PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Last, PCR products were purified (again, using the AMPure XP system) and the library quality was assessed on an Agilent Bioanalyzer 2100 system. Libraries were sequenced on an Illumina platform (125 bp/150 bp paired-end read lengths). Raw reads were processed through Novogene perl scripts.

Novogene Corp. performed preliminary informatics by first cleaning reads, removing reads containing adapters, reads containing poly-N, and low-quality reads (uncertain nucleotides > 10%, sQ ≤ 20% for > 50% of reads) from raw data and calculating Q20, Q30 and GC content from the clean data. All the downstream analyses were based on the clean, high quality data. They aligned reads to the *G. aculeatus* reference genome (Ensembl release 94) using HISAT2 v2.1.0.

*Differential Gene Expression Analysis*

We conducted gene expression analyses using R v3.5.3 (The R Foundation for Statistical Computing, 2019) and the Bioconductor v3.8 R package edgeR v3.24.3 (Robinson, McCarthy, & Smyth, 2010). We included genes with at least 1 read per

kilobase million (RPKM) in at least 3 samples in our analyses. We calculated normalization factors based on library sizes and estimated dispersion (Chen, Lun, & Smyth, 2014). To assess differential gene expression, we used a negative binomial generalized linear model (design = treatment) using *glmQLFit* and defined contrasts to perform pairwise comparisons between treatments (Lun, Chen, & Smyth, 2016). To produce heatmaps, we made planned contrasts between all parental effects and parental effects plus direct exposure treatments and the no exposure control and used default clustering in *pheatmap* (Kolde, 2019) to determine similarities in gene expression profiles among contrasts. We defined particular contrasts to answer our two questions. To address whether gene expression varied with indirect cues (maternal, paternal, and joint parental effects), we compared gene expression in the maternal effects treatment, the paternal effects treatment, and the joint parental effects treatment each to the no exposure control in a heatmap. We additionally determined the number of expressed genes, shared and unique, among all pairwise groups in this heatmap (rpkm cutoff = 1). To address whether gene expression of offspring depends on interactions between predator-induced parental effects *and* direct predator exposure (whether or not parent and offspring environments matched or mismatched), we produced a heatmap showing pairwise contrasts between each parental effects only treatment and the no exposure control and each parental effects plus direct exposure treatment and the no exposure control. Finally, we used *decideTestsDGE* ($p = 0.05$) to determine the number of significant differentially expressed genes between all possible pairwise comparisons of the eight treatments in Figure 3.1.

## RESULTS

We recovered an average of 36.8 million clean reads per sample (total genes pre-filtering = 22,456; total genes post-filtering = 18,430). Overall, gene expression patterns appear to depend on parental predator-exposure. The gene expression profiles of offspring resulting from maternal and paternal predator-exposure were more similar to each other than they were to offspring from jointly exposed parents (Figure 3.2A). This is particularly clear on the bottom half of the heat map. In support of this pattern, the maternal and paternal effects treatments shared substantially more expressed genes with the no predator exposure control (N = 1494) than were shared among the maternal, paternal, and joint parental effects treatments (N = 50) (Figure 3.2C). There were also some genes uniquely expressed in the maternal effects and paternal effects treatments (N = 163 genes, N = 181 genes, respectively; Figure 3.2C). Testing for differentially expressed genes in contrasts of maternal or paternal effects with the no exposure control revealed there were no significantly differentially expressed genes in either of these pairings, but 1,256 genes were significantly differentially expressed in the contrast of joint parental effects with the no exposure control ($N_{up}$ = 544, $N_{down}$ = 712) (Figure 3.3). Finally, there were more differentially expressed genes between the maternal effects and joint parental effects treatments ($N_{up}$ = 273, $N_{down}$ = 10) than between the paternal effects and joint parental effects treatments ($N_{up}$ = 3,$N_{down}$ = 0) (but none between maternal and paternal effects; Figure 3.3).

Whether or not parent and offspring environments matched (offspring also experienced direct predator risk) or mismatched (offspring had no direct predator experience) had little influence on differential gene expression patterns. Regardless of the

source of parental predator-exposure, single-parent or joint, differential gene expression patterns under parental effects only and parental effects plus direct predator exposure were similar (Figure 3.4A), and in fact, there were no significantly differentially expressed genes between each of the parental effects only treatments and their respective parental effects plus direct exposure treatments (Figure 3.3). When comparing the parental effects plus direct exposure treatments to each other, numbers of significantly differentially expressed genes varied with the source of parental predator-exposure (maternal, paternal, or joint). For instance, we found few differentially expressed genes between the paternal effects only and joint parental effects only treatments but found substantial differential expression between the paternal effects plus direct exposure and joint parental effects plus direct exposure treatments (Figure 3.3). This suggests that direct exposure interacts with indirect cues to produce variation in gene expression but not sufficiently enough to detect differences between parental effects only treatments and parental effects plus direct exposure treatments. There were more differentially expressed genes when comparing the paternal effects plus direct exposure to the joint parental effects plus direct exposure treatments than when comparing the maternal effects plus direct exposure to either the paternal effects plus direct exposure or joint parental effects plus direct exposure (Figure 3.3), suggesting that there is something special about joint parental effects plus direct exposure; it seems to produce more dramatic changes in gene expression than maternal effects plus direct exposure and (especially) paternal effects plus direct exposure.

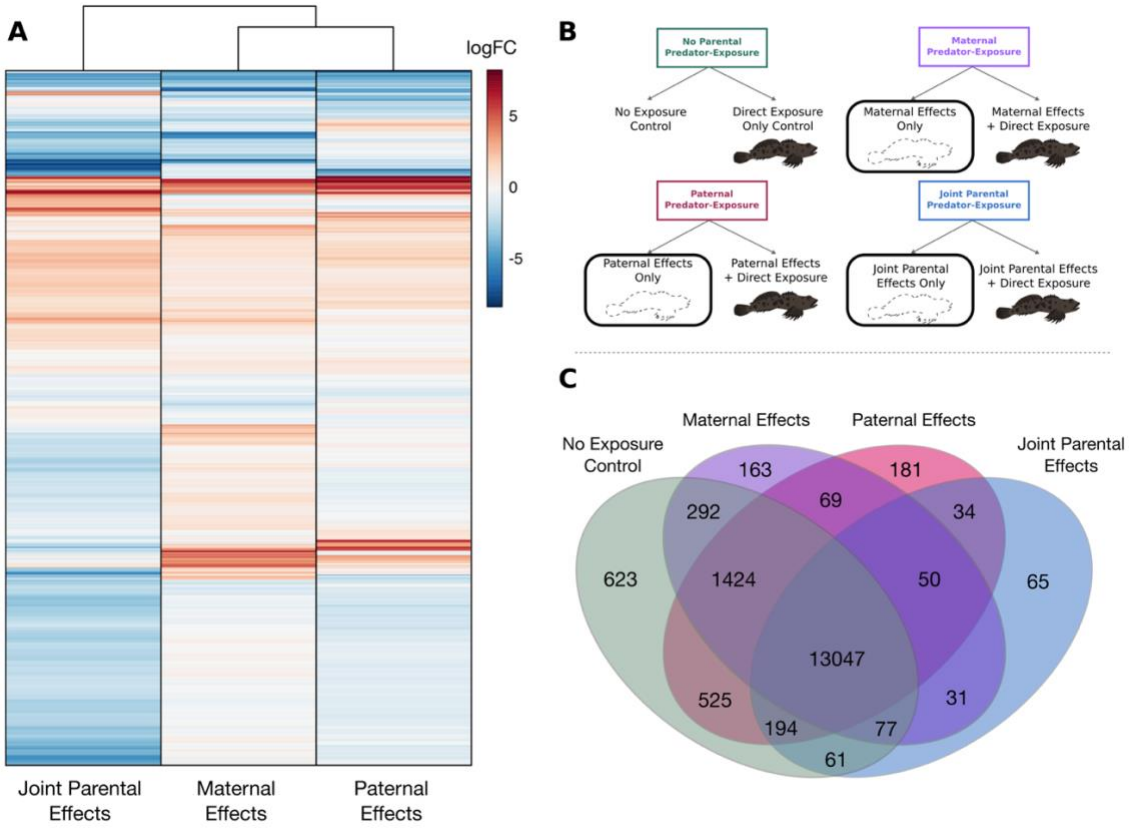


**Figure 3.2. Patterns of gene expression with single-parent versus joint parental predator-exposure.** (A) Heatmap showing the differential expression patterns of the 500 most differentially expressed genes among all contrasts. Columns are pairwise contrasts relative to the "No Exposure Control" treatment. The dendrogram above the heatmap identifies similarities in differential gene expression among groups. Red = upregulated genes, Blue = downregulated genes. (B) Diagram indicating which treatments are compared in this heatmap (these are a specific subset of the whole experimental design, focused on gene expression following from parental effects in the absence of direct predator exposure to offspring; treatments included in the heatmap are outlined in black). (C): Venn diagram showing the number of shared and unique expressed genes (both up- and downregulated) among groups using RPKM values.

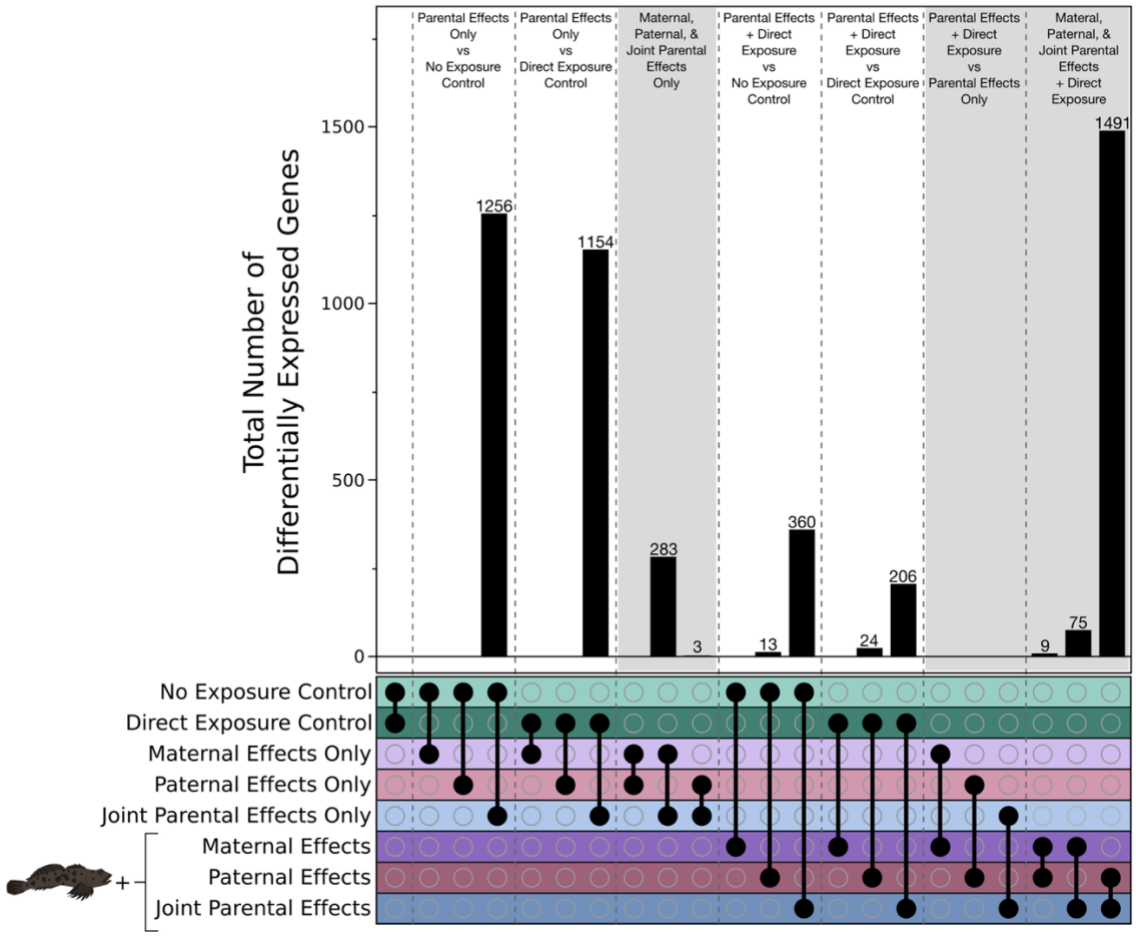


**Figure 3.3. Total number of differentially expressed genes per contrast.** The bar graph (top) shows the total number of differentially expressed genes in a given contrast. Immediately below each bar is a table showing which contrast is shown in each column. A pair of black circles connects the two treatments being compared in each contrast. Columns highlighted in grey are contrasts that directly address our two main questions: 1) how does gene expression vary with maternal, paternal, and joint parental predator-exposure (grey column at left) and 2) how does gene expression vary with parental predator-exposure when parent and offspring environments match and mismatch (two grey columns at right)?

## DISCUSSION

We first considered whether and how gene expression in the diencephalon differs when offspring receive indirect predator cues through maternal, paternal, and joint parental predator-exposure. We found intriguingly that maternal effects and paternal effects on offspring gene expression profiles were similar to one another, but that both appear to differ from joint parental effects on offspring gene expression (Figure 3.2, 3.3).

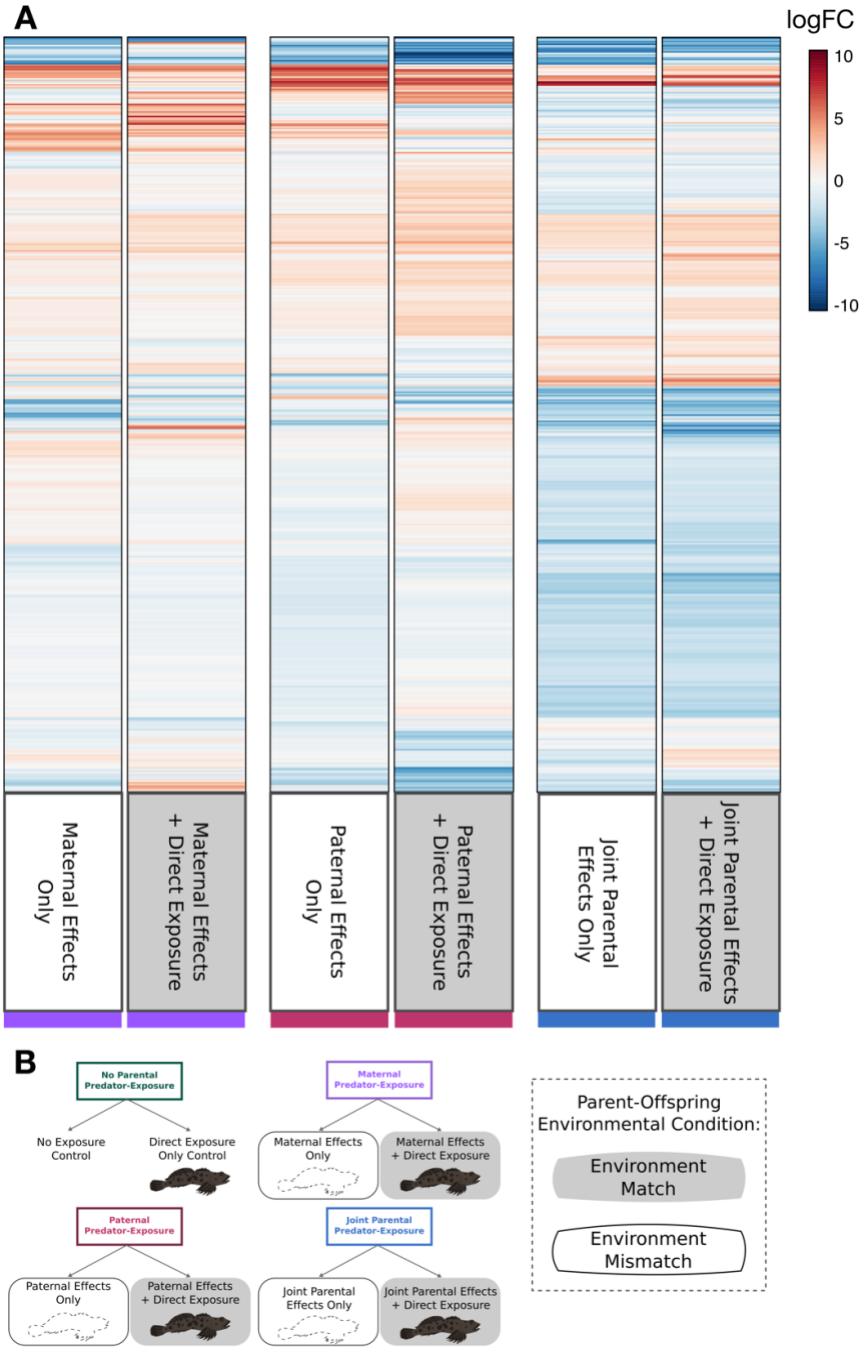


**Figure 3.4. Differential gene expression when parent and offspring environments match and mismatch.** (A) Heatmap showing the differential expression patterns using the 500 top-most differentially expressed genes among all contrasts. Each column is a pairwise contrast to the "No Exposure" control. Red = upregulated genes, Blue = downregulated genes. The shading behind treatment names represent parent-offspring environmental conditions (environmental match or mismatch), and colors below treatment names indicate parental predator-exposure. (B) Diagram indicating which treatments are compared in the heatmap within the experimental design (treatments with black outline or grey fill).

Our differential gene expression analysis supports this finding in that only joint parental effects produced detectable differences in gene expression when compared to the no exposure control. This finding further suggests that maternal and paternal effects may act additively on offspring gene expression. It is possible that paternal effects mediate the differences in gene expression we observe with joint parental effects, as we observed more differentially expressed genes when comparing maternal and joint parental effects than when comparing paternal and joint parental effects (i.e. gene expression profiles from paternal and joint parental effects were more similar; Figure 3.2A, 3.3).

Though we cannot say that the differentially expressed genes found here underlie the variation in behavior and physiology we previously uncovered, it is interesting to note that we found similar patterns in the differences between maternal, paternal, and joint parental effects on offspring behavior in our previous work (Lehto and Tinghitella, in revision). We found that maternal and paternal predator-exposure produced similar changes in mating behavior, both reducing responsiveness and relaxing mating preferences, but the effects of joint parental predator-exposure on daughters' behavior differed from those of single-parent exposure. In two studies examining predator-induced maternal and paternal effects on offspring gene expression separately, maternal predator-exposure impacted embryo size and gene expression (Mommer & Bell, 2014), and paternal predator-exposure impacted juvenile offspring size and gene expression (Stein et al., 2018). Here, by examining maternal and paternal effects in the same study, we can answer additional questions about whether those changes in gene expression following from maternal and paternal effects are similar. We detected no differentially expressed genes between maternal and paternal effects treatments, despite dramatic differences in

the ways that males and females contribute to offspring development and the manner in which they were exposed to the model predator to generate parental effects. However, when we contrasted single-parent effects with joint parental effects, each comparison revealed differentially expressed genes. A possible explanation for this pattern is that changes in gene expression may be mediated through parental care. Male sticklebacks are known to reduce their parental care behavior both when they experience direct predation risk *and* when they are mated with predator-exposed females (Lehto & Tinghitella, in revision; McGhee et al., 2015), so paternal care may be further reduced when both parents experience predation risk. If paternal care influences gene expression profiles (Fish et al., 2010; McGhee & Bell, 2014), then, predator-induced maternal and paternal effects might produce similar gene expression profiles (and phenotypes), but under, joint parental effects, gene expression might differ more substantially.

When we compared the gene expression of offspring who received indirect predation cues through parental effects only and offspring who received both indirect cues and had direct experience with the predator model, we found no differentially expressed genes, regardless of the source of parental effects (maternal, paternal, or joint parental). That is, gene expression was the same when parent and offspring environments matched and mismatched. It is possible that, as in Stein et al. (2018), when indirect cues from parents are combined with direct exposure, the effects on offspring expression and phenotypes are redundant rather than additive (more dramatic). This would be possible if, for instance, offspring responses to predation were threshold traits, and indirect cues via parental effects were sufficient to reach the threshold required to express anti-predator traits (Buoro, Gimenez, & Prévost, 2012; McCollum & Van Buskirk, 1996). Interestingly

though, we also found no differences in gene expression between the direct exposure and no exposure controls. Therefore, the lack of differential gene expression in matching versus mismatching parent and offspring environments may be partially due to the direct predator exposure regime we used in this experiment. Perhaps indirect cues generated through parental effects impact offspring responses to acute predator attack rather than the chronic, predation risk conditions we mimicked in the direct exposure (Ellison & Ydenberg, 2019; Elvidge, Ramnarine, & Brown, 2014; Ferrari et al., 2009). If so, a more punctuated-predator exposure with more immediate sampling might reveal variation in offspring gene expression when parent-offspring predation environments match and mismatch.

There are several other possible reasons that direct predator exposure might not have impacted gene expression patterns. One of those is the age of the fish at the time of direct predator exposure. Our stickleback were approximately two years old when they were exposed to the sculpin model. The only other paper that has considered whether direct predator exposure and predator-induced parental effects induce similar gene expression responses looked at impacts of direct predator exposure on 2-3 month-old stickleback (Stein et al. 2018). The age of the fish when predator-exposed could be particularly relevant if sculpin are more often predators of eggs and juvenile fish than they are adults (Foster, 2010). If so, sculpin exposure may be a more important ecological force for juvenile stickleback and adult stickleback that are reproductive than it is for non-reproductive adults. Also, the stickleback used in our study, having been lab reared and maintained for more than two years, may have been habituated to general disturbances relating to husbandry, rendering the predator model insufficient to generate

stressful conditions that influence gene expression. Finally, stickleback (Candolin, 1998), moths (Lafaille, Bimbard, & Greenfield, 2010), and black gobies (Magnhagen, 1990), reduce their predator avoidance behaviors to maximize mating opportunities as adults. Though our fish were not in reproductive condition (breeding coloration for males or gravidity for females) at the time of direct predator exposure, if adults are less risk averse, this may explain why the direct simulated predation risk did not influence gene expression in this case.

Here we show that predator-induced maternal, paternal, and joint parental effects influence offspring gene expression: maternal and paternal effects produced similar variation but jointly produce dramatic shifts in offspring gene expression. In systems where both parents contribute substantially to offspring development, maternal, paternal, and joint parental ecological experiences may contribute to immense variation in offspring phenotypes. Much of the recent attention paid to parental effects asks whether or not they might allow for rapid offspring responses that precede and facilitate adaptive evolution in changing environments (Bonduriansky, Crean, & Day, 2012; Nettle & Bateson, 2015; Uller, 2008; Uller et al., 2013). Though offspring gene expression was not altered by the addition of direct predator experience in our study, that we find changes in offspring gene expression with parental predator-exposure more generally points to potentially robust epigenetic transgenerational changes that may underlie a multitude of other offspring characteristics.

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# Appendix

## Supplementary Information for

Chapter One: Predator-induced maternal and paternal effects independently alter sexual selection

*This appendix includes:*

Supplementary methods
SI reference citations
Figure S1
Tables S1 to S3

**Supplementary Methods:**

*Quantification of Male Nuptial Coloration and Sexual Signals PCAs*

We quantified the red throat area and blue eye area of wild-caught males used in no-choice trials from photographs taken immediately before and immediately after each no-choice mating trial. All photographs were taken with a digital camera (Canon PowerShot G15) under standardized lighting (four xenon, 20 W bulbs) inside a photobox that held the camera and blocked ambient light. In each photo the fish was on its right side against a neutral background with a millimeter ruler in view for scale. We measured red throat area as a proportion of total body area in FIJI (Schindelin et al. 2012). For each photograph, we selected red coloration across the whole body using the Threshold Color plugin (Y = 32-255, U = 0-143, V = 141-255; following (Wong et al. 2007; Tinghitella et al. 2018)) and determined total body area using the SIOX: Simple Interactive Object Extraction. To measure the blue area of the eye, we drew a circle that encompassed the eye (175 x 175 pixels) in FIJI and selected blue coloration (Threshold Color plugin; Y = 25-255, U = 123-255, V = 0-141). We scaled each color area using the millimeter ruler, determining red area as a proportion of total body size and blue area as a proportion of the standard 175x175 pixel circle.

We then used PCA as a variable reduction technique to obtain a single measure of male sexual signals. For each color measure (red throat and blue eye), we first obtained the residuals of a regression of after-photo color area onto before-photo color area in JMP 12.0 to account for the plasticity in male coloration between the start and end of laboratory courtship trials. We then scaled the two color measurements by regressing red

throat area onto blue eye area to obtain a single color measure. Blue eye and red throat color were measured on different scales, so without scaling, blue eye could have dominated PCs when using covariances to construct the principal components. We then used PCA to combine male color and courtship behaviors. For each stage of courtship analyzed, the PCA included male color and all of the male behaviors that occur at that stage of courtship (Table S2). For example, when assessing the female follow stage, the Male Signals PCA included male color and the following behaviors: male approaches, zig-zags, bites, and leads (Table S2). All PCAs were performed in JMP 12.0. For each stage of courtship, the first principal component (PC1) explained 54-59% of variation. Male color and zig-zags loaded most strongly onto PC1. Higher values of each Male Signals PC1 described males with greater red throat and blue eye area who performed more conspicuous zig-zag behaviors. We would expect these particular signals to be correlated as the zig-zag movements of a male accentuate his red throat (Rowland 1984). The remaining male courtship behaviors loaded strongly onto PC2, which explained 25-27% of variation.

**Supplementary References:**

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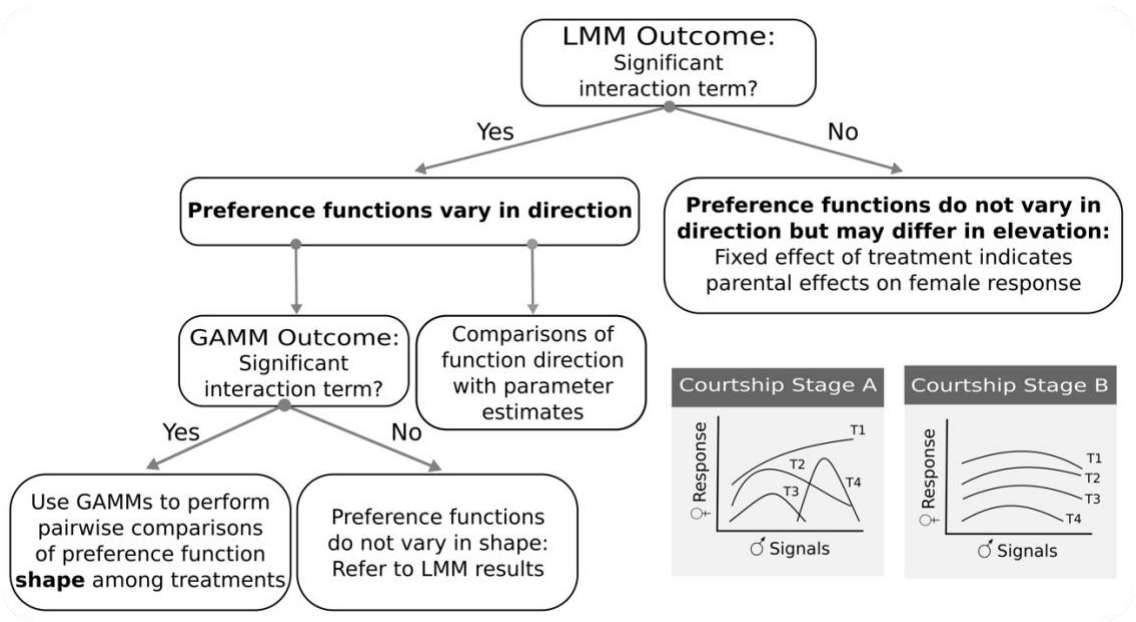


**Figure S1. Testing for differences in preference function direction and shape using LMMs/GLMMs and GAMMs.** We characterized the direction and shape of female preference functions and tested for parental effects on each of the four distinct stages of courtship allowing us to assess parental effects on interest in mating (early courtship), preference (follow and examine), and mate choice (enter nest). For each courtship stage, we first constructed treatment-level preference functions in PFunc (Kilmer et al., 2017). In the bottom right, T1 - T4 indicate four treatments for which preference functions were measured at two different courtship stages, A and B. Then, for each courtship stage, we used linear mixed model and generalized linear mixed model (LMM/GLMM) analyses to determine whether daughters' mating behavior depended on an interaction between treatment and male sexual signals as shown in Courtship Stage A. When an LMM/GLMM produced a significant interaction term indicating differences in preference function direction, we used generalized additive mixed models (GAMMs) to confirm differences in preference function shape among treatments (significant interaction term) and when both GAMM and LMM/GLMM models agreed (both indicated significant interaction terms), then, and only then, we used GAMMs to further probe pairwise treatment-by-treatment differences in preference function shape. When LMMs/GLMMs did not indicate a significant interaction between parental predator exposure and male sexual signals, we looked for a fixed effect of parental predator exposure on daughters' mating behavior, indicating potential differences in preference function height but not shape as shown in Courtship Stage B.

**Table S1.** Descriptions of courtship behaviors (adapted from Tinghitella, Lehto, et al., 2015).

| **Courtship Behavior** | **Description** |
|---|---|
| *Female* | |
| Approach | Movement towards male to within a body length |
| Angle | Female's body at 45$^{o}$ incline |
| Head-up | Swift motion into an "Angle" |
| Follow | Trails male after a "Lead" |
| Examine Nest | Moves nose near entrance of nest |
| Enter Nest | Moves into nest and ceases movement inside of nest |
| *Male* | |
| Approach | Movement towards female to within a body length |
| Zig-zag | Quick left-right movements |
| Bite | Nips female with mouth |
| Lead | Directs female towards nest |
| Show | Gestures to nest entrance with nose and body nearly on its side |
| Rub | Pushes with ventral side on female's dorsal side as female examines or enters nest |

**Table S2.** Factor loadings for all principal components analyses performed for variable reduction of male signals (color measures and courtship behavior) and preceding female courtship behaviors. All behaviors in PCs were scaled for the duration of the no-choice mating trial prior to PCA. A '-' for a given behavior indicates that it was excluded from a PCA because it occurs in a later stage of courtship.

| **Courtship Stage:** | *Early Courtship* | *Follow* | *Examine Nest* | *Enter Nest* |
|---|---|---|---|---|
| **Male Signals** | PC1 | PC1 | PC1 | PC1 |
| Eigenvalue | 0.0029 | 0.0029 | 0.0029 | 0.0029 |
| Variance explained | 0.585 | 0.554 | 0.546 | 0.535 |
| *Factor loadings* | | | | |
| Male color* | 0.995 | 0.992 | 0.991 | 0.991 |
| Male approach | 0.220 | 0.253 | 0.256 | 0.260 |
| Zig-zag | 0.317 | 0.325 | 0.326 | 0.327 |
| Bite | 0.088 | 0.125 | 0.128 | 0.133 |
| Lead | - | 0.289 | 0.292 | 0.296 |
| Show | - | - | 0.155 | 0.160 |
| Rub | - | - | - | 0.122 |
| **Preceding Female** | PC1 | PC1** | PC1 | PC1 |
| Eigenvalue | - | 0.0003 | 0.0005 | 0.0005 |
| Variance explained | - | 0.833 | 0.795 | 0.742 |
| *Factor loadings* | | | | |
| Female approach | - | 0.997 | 0.976 | 0.963 |
| Angle | - | 0.640 | 0.641 | 0.623 |
| Head-up | - | 0.495 | 0.502 | 0.511 |
| Follow | - | - | 0.881 | 0.899 |
| Examine | - | - | - | 0.639 |
| Enter | - | - | - | - |

*Male color is a combined measure of male red throat area and blue eye area (see supplementary methods above).
**The PC1 comprised of early courtship behaviors at the Follows stage is the same PC1 that was the outcome variable in models examining female early courtship behavior.

**Table S3.** We compared generalized additive mixed models (GAMMs) built with a single smoother (A) to GAMMs built with multiple smoothers (B) to determine whether parental effects on offspring behavior changed the shape of preference functions at the follows stage. A courtship stage best modeled with multiple smoothers indicates differences in preference function shape among treatments as determined by AIC. By examining the smoother on a given treatment within one courtship stage in a GAMM with multiple smoothers, we can determine whether the preference function is 'open' (linear) or 'closed' (non-linear/curvier) (e.g. At the follow stage the smoothers on predator-exposed mother and predator-exposed father female offspring are significantly non-linear or 'closed'.)

**A. Single Smoother GAMM** **AIC = -483.98, df = 12.20**

| ***Parametric Terms*** | *Estimate* | *SE* | *t* | *P* | ***Smooth Terms*** | *edf* | *Ref.df* | *F* | *P* |
|---|---|---|---|---|---|---|---|---|---|
| Male Length (mm) | -0.0005 | 0.0003 | -1.39 | 0.17 | *Random Effects* | | | | |
| Preceding Female Behaviors (PC1) | 0.52 | 0.05 | 10.14 | 1.6E-14 | Family nested in female treatment | 4.17 | 15.00 | 0.425 | 0.13 |
| | | | | | Male ID | 2.32E-5 | 27.00 | 0.00 | 0.50 |
| *Female Treatment* | | | | | Male Signals (PC1) | 1.03 | 1.06 | 10.66 | 0.00 |
| Control | 0.04 | 0.02 | 2.12 | 0.04 | | | | | |
| Predator-Exposed Mother | 0.04 | 0.02 | 2.02 | <0.05 | | | | | |
| Predator-Exposed Father | 0.04 | 0.02 | 2.06 | 0.04 | | | | | |
| Predator-Exposed Parents | 0.04 | 0.02 | 2.25 | 0.03 | | | | | |

**B. Multiple Smoother GAMM** **AIC = -490.94, df = 16.75**

| ***Parametric Terms*** | *Estimate* | *SE* | *t* | *P* | ***Smooth Terms*** | *edf* | *Ref.df* | *F* | *P* |
|---|---|---|---|---|---|---|---|---|---|
| Male Length (mm) | -0.0004 | 0.0003 | -1.19 | 0.24 | *Random Effects* | | | | |
| Preceding Female Behaviors (PC1) | 0.50 | 0.05 | 10.35 | 1.89E-14 | Family nested in female treatment | 5.18 | 15.00 | 0.53 | 0.09 |
| | | | | | Male ID | 4.21E-6 | 27.00 | 0.00 | 0.82 |
| *Female Treatment* | | | | | *Male Signals (PC1) by:* | | | | |
| Control | 0.04 | 0.02 | 1.99 | 0.05 | Control | 0.89 | 1.06 | 0.14 | 0.68 |
| Predator-Exposed Mother | 0.03 | 0.02 | 1.84 | 0.07 | Predator-Exposed Mother | 0.63 | 0.65 | 14.76 | 0.003 |
| Predator-Exposed Father | 0.03 | 0.02 | 1.89 | 0.06 | Predator-Exposed Father | 0.62 | 0.64 | 10.31 | 0.01 |
| Predator-Exposed Parents | 0.04 | 0.02 | 2.08 | 0.04 | Predator-Exposed Parents | 0.61 | 0.62 | 1.13 | 0.41 |