Sex-specific transgenerational plasticity in threespined sticklebacks

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Abstract (250 words)

Sex-specific selection pressures can generate different phenotypic optima for males and females in response to the current environment, i.e. sex differences in phenotypic plasticity. Less widely appreciated is the possibility that transgenerational plasticity (TGP) can also depend on sex. Sex-specific TGP is potentially of great evolutionary significance, as it is a mechanism by which mothers and fathers can exert different effects on offspring traits and by which potentially adaptive traits can persist selectively across generations via only daughters or sons. Here, we demonstrate that maternally- and paternally-mediated TGP in response to predation risk have largely distinct effects on offspring traits in threespined sticklebacks (*Gasterosteus aculeatus*). Predator-exposed fathers produced sons that were more risk-prone, while predator-exposed mothers produced more anxious sons and daughters. Further, when combined together, maternal and paternal environments on offspring survival were nonadditive. Such sex-specific effects could occur if predation risk causes mothers and fathers to activate different developmental programs in sons versus daughters. Consistent with this hypothesis, offspring brain gene expression profile depended on whether their mother and/or father had been exposed to risk, and the influence of maternal and paternal environments varied between male and female offspring. Altogether these results draw attention to the potential for sex to influence patterns of TGP, and raise new questions about the evolution of plasticity at the interface between sexual conflict and parent-offspring conflict, with paternal strategies, maternal strategies, and offspring counter-adaptations all ultimately dictating offspring phenotypes.

Key words: maternal effect, paternal effect, *Gasterosteus aculeatus*, phenotypic plasticity, intergenerational plasticity, nongenetic inheritance

Significance

TGP helps organisms cope with environmental change by bridging the gap between within-generational plasticity and long-term evolutionary change. Sex-specific TGP may allow mothers and fathers to selectively alter the phenotypes of their sons and daughters in response to the environment with a greater degree of precision than genetic inheritance and in ways that match the distinct life-history strategies of males and females. By isolating cues coming from mothers versus fathers and separately evaluating phenotypic effects in sons versus daughters, we show that interactions between maternal cues, paternal cues, and offspring sex are integral to understanding when and how the past environment influences future phenotypes, and the conditions that favor the evolution of TGP.

Introduction

Sex differences in life-histories (e.g. reproductive lifespan, mortality rate) or reproductive strategies can favor different optimal phenotypes in males and females (1). Although a shared genetic basis can constrain phenotypic differences between the sexes (2, 3), epigenetic changes can overcome this constraint and allow males and females to respond differently to the same environmental condition (within-generational plasticity). Sex-specific patterns of within-generational plasticity have been documented in diverse taxa (4-7); for example, in cichlids, predation risk experienced early in life influenced the development of males, but not females, likely because males are more vulnerable to predation (5).

While less explored, there also is evidence for sex-specific ***transgenerational*** plasticity (TGP; also referred to as intergenerational plasticity); specifically, that the sex of the parent and/or the offspring can alter the ways in which environments encountered by recent ancestors affect future generations. For example, maternal versus paternal exposure to the same environmental condition can have different effects on offspring (8-10) and the influence of parental cues (whether mediated by the mother or the father) often depends on the sex of the offspring (11-17). Despite the biological reality that they probably often co-occur, we know little about the potential for interactions between maternal cues, paternal cues, and offspring sex during TGP because they are typically studied in isolation of each other. However, the influence of maternal cues might depend on paternal cues (or vice versa) (9, 18), and/or offspring might selectively respond to information from one parent over the other (e.g., daughters respond to maternal cues while sons respond to paternal cues).

Understanding the ways in which the maternal cues, paternal cues, and offspring sex interact during TGP could help clarify evolutionary phenomena such as sexual conflict, parent-

offspring conflict, and genomic imprinting, which is thought to arise from sexual conflict over resource allocation to offspring. For example, sexual conflict may cause mothers and fathers to favor different phenotypes in their offspring, resulting in the evolution of mechanisms that allow mothers to manipulate the ways in which fathers influence offspring (e.g. via cytoplasmic contributions (19)) or fathers to manipulate the ways in which mothers influence offspring (e.g. via ejaculate composition (20)). Further, nongenetic inheritance that functions in a sex-specific manner can resolve evolutionary conflicts that occur when selection favors different phenotypes in sons and daughters (21) because male versus female offspring may integrate parental information in ways that match their distinct life history trajectories. One way that such sex-specific inheritance could operate is if cues from mothers and fathers activate different developmental programs in daughters and sons.

Here, we evaluate sex-specific TGP in threespined sticklebacks (*Gasterosteus aculeatus*). Male and female sticklebacks are sexually dimorphic in several respects, including in habitat use (22), diet (23), parasite load (23), and morphology (24), with these differences beginning to emerge during early adulthood (22, 23). Sticklebacks also have a variety of male-specific reproductive traits that increase male vulnerability to predation risk (25, 26): male sticklebacks develop bright nuptial coloration, engage in conspicuous territory defense and courtship behavior, and are the sole providers of paternal care that is necessary for offspring survival (27). These sex differences in behavior and life history often expose males and females to different predation regimes (28), likely altering the environment experienced by mothers versus fathers and the optimal phenotype for daughters versus sons in response to predation risk.

We exposed adult male and female sticklebacks to simulated predation risk prior to fertilization and used a fully factorial design to generate offspring of control (unexposed)

parents, offspring of predator-exposed mothers, offspring of predator-exposed fathers, and offspring of predator-exposed mothers and fathers. Because predation risk varies in both space and time, it is likely that there is a mix of reproductively mature males and females who either have or have not recently experienced predation risk within natural populations. We reared offspring under 'control' conditions (i.e. in the absence of predation risk) and then compared traits relevant to predator defense between sons and daughters, including survival against live predators, and used brain gene expression data to gain insights into the underlying mechanisms.

Results

Sons, but not daughters, of predator-exposed fathers were more risk-prone

We compared maternal and paternal exposure to predation risk on the risk-taking behavior of sons and daughters. We used an open field assay to measure offspring activity/exploration and boldness under baseline conditions and after a simulated predator attack. Offspring were significantly less active/exploratory after the simulated predator attack compared to before (principal component analysis: higher values indicate more active and explorative individuals; MCMC GLMM: 95% CI (-1.24, -0.70), $p<0.001$), confirming that offspring behaviorally responded to personally-experienced risk. There was a significant interaction between paternal treatment and offspring sex on offspring activity/exploration (pooled before and after the simulated predator attack: 95% CI (0.25, 1.54), $p=0.005$; Figure 1A). Specifically, sons of predator-exposed fathers were significantly more active/exploratory compared to sons of control fathers (95% CI (-1.30, -0.20), $p=0.01$), but there was not a detectable effect of paternal treatment on female offspring (95% CI (-0.40, 0.81), $p=0.49$). This suggests that sons were especially responsive to paternal exposure to predation risk. Greater activity in response to

exposure to predation risk is consistent with higher risk-taking behavior observed in sticklebacks from high predation populations compared to low predation populations (29).

We did not detect a significant effect of maternal or paternal treatment on boldness (principal component analysis: higher values indicate less bold fish with an increased latency to emerge from the shelter and to resume movement after the predator attack), although female offspring were less bold than male offspring (SI Appendix Table S1). We found no significant effect of maternal or paternal predation exposure on offspring standard length or body mass at 4.5 months, nor did they vary between male and female offspring; however, larger fish were less active/exploratory and less bold (SI Appendix Table S1).

Offspring of predator-exposed mothers, but not fathers, were more cautious

Scototaxis – preference for dark – is often associated with increased cautiousness, or anxiety-like behavior (30). In order to determine whether a parent's experience with predation risk influences the anxiety-like behavior of their offspring (31), we conducted light-dark preference tests in a half-black/half-white tank. Offspring of predator-exposed mothers were more cautious (principal component analysis: took longer to enter the white area, spent less time in the white area, and switched less between black and white areas) compared to offspring of control mothers (MCMC GLMM: 95% CI [0.06, 1.09], p=0.03; Figure 1B). However, we did not detect an effect of paternal treatment on offspring scototaxis behavior (95% CI [-0.79, 0.32], p=0.44). Both female (95% CI [-1.27, -0.17], p=0.01) and smaller (95% CI [-0.10, -0.006], p=0.03) offspring showed more cautious behavior. We found no evidence of seasonal effects (experimental day: 95% CI [-0.004, 0.01], p=0.33).

142 ***Mothers mitigated the fitness costs of paternal exposure to predation risk***

143 To understand if parents' experience with predation risk altered offspring survival in an
144 encounter with a predator (reviewed in (32)), we measured offspring survival against live sculpin
145 predators as well as offspring response to an acute stressor (confinement stress). There was a
146 significant interaction between maternal and paternal treatment on offspring survival in live
147 predation assays (generalized linear mixed effect model: Z_{335} = -1.98, 0.047). Specifically,
148 offspring of predator-exposed fathers were more frequently captured by the predator compared to
149 offspring of control parents, but this was not true for offspring of predator-exposed mothers or
150 both a predator-exposed mother and father (Figure 1C). This suggests that was a strong fitness
151 cost of having a predator-exposed father, but mothers seemed to mitigate those costs, perhaps by
152 making their offspring more cautious (see above). Survivors of the successful predation trials
153 were heavily female biased (93/148; Chi-squared: χ^2=9.76, p=0.002), suggesting that males are
154 generally more vulnerable to predation risk. The sex-bias was not significantly different across
155 treatment groups (χ^2=3.03, p=0.39). We found no effect of size on how frequently the
156 stickleback were captured by the predator (Z_{335} = 1.56, 0.11). Further, we did not find evidence
157 that parental treatment significantly altered offspring stress response (opercular beat rate),
158 although lower opercular beat rate tended to be correlated with reduced likelihood of survival in
159 the predation assays (SI Appendix).
160
161 ***Distinct and nonadditive effects of maternal and paternal treatment on offspring brain gene***
162 ***expression***
163 The results described above suggest that predation risk experienced by mothers versus
164 fathers has very different consequences for offspring development. In order to evaluate this

question at the molecular level, we used pair-wise contrasts to compare the baseline brain gene expression profile of offspring of unexposed parents (control) to offspring of predator-exposed mothers, predator-exposed fathers, and two predator-exposed parents in male and female offspring separately. We found that the effects of maternal and paternal treatment on brain gene expression were approximately equivalent in magnitude and were largely nonoverlapping (Figure 2A,B): in sons, for example, 1028 genes were differentially expressed in response to maternal experience with risk, 904 genes were differentially expressed in response to paternal experience with risk while only 253 genes were shared between them (daughters show a similar pattern, Figure 2A). Interestingly, there was also a large number of genes that were unique to the "both" condition, i.e. between offspring of two predator-exposed parents versus the control; these differentially expressed genes could reflect the ways in which maternal and paternal cues interact at the molecular level.

Of the differentially expressed genes that were shared between the pairwise comparisons, nearly all were concordantly regulated, for both sons and daughters (Figure 2A,B). This suggests that, despite the large-scale differences in brain gene expression between offspring of predator-exposed mothers and fathers, there is a core set of genes that is activated in offspring brains in response to either maternal or paternal exposure to predation risk.

Maternal and paternal exposure to predation risk interacted with offspring sex to influence offspring brain gene expression

The behavioral data suggest that sons and daughters respond to parental experience with predation risk differently, with sons, but not daughters, increasing activity/exploration in response to paternal experience with predation risk. One way that such sex-specific inheritance

could arise is if cues from one parent (e.g. fathers) activate a particular developmental program in one offspring sex but not the other (e.g. in sons but not daughters).

To test this hypothesis, we used WGCNA to identify clusters ("modules") of genes with similar expression patterns. This procedure reduces the dimensionality of the transcriptomic dataset, which allowed us to explore the potential for interactive effects of maternal treatment, paternal treatment and offspring sex on modules of genes with correlated expression patterns. WGCNA identified 23 informative modules (or clusters of genes with coordinated expression) in the dataset. The expression of eight of the 23 modules was significantly affected by at least one of the factors in the model: three modules were significantly affected by maternal treatment, two were significantly affected by the two-way interaction between maternal and paternal treatment, and three were significantly affected by the three-way interaction between paternal treatment, maternal treatment and offspring sex (shown in Figure 2C). For example, the module "saddle brown" comprises 48 co-expressed genes (largely enriched for developmental processes) whose expression was influenced by the three way interaction between maternal treatment, paternal treatment and offspring sex. Specifically, daughters of predator-exposed mothers or fathers showed lower expression of genes in this module compared to daughters of control parents or two predator-exposed parents (Figure 2C). For sons, on the other hand, the expression of genes in this module was more strongly affected by maternal treatment. A similar pattern was observed in the yellow and cyan modules. Overall these results demonstrate that at the molecular level, daughters and sons differ in the extent to which they respond to predation risk that had been experienced by their mother, father or by both parents.

Discussion

211 Transgenerational plasticity allows environmental information to be delivered to
212 offspring earlier and with potentially lower costs to offspring than developmental plasticity,
213 which may allow offspring to develop traits during early development that help them cope with
214 environmental change (33, 34). Unlike genetic inheritance, TGP can potentially be fine-tuned to
215 the precise environment that offspring will encounter (21). In particular, males and females often
216 experience different environments because of sex differences in life history and reproductive
217 strategies, and TGP might allow parents to confer different phenotypes to sons and daughters.
218 The results reported here draw attention to the importance of sex-specific TGP: offspring
219 phenotypes varied depending on whether predation risk had been experienced by their mother or
220 their father, and a parent's experience with predation risk produced different phenotypes in their
221 sons compared to their daughters.
222 We find that maternal and paternal exposure to the same environmental factor (predation
223 risk) generated largely distinct effects in offspring: predator-exposed mothers produced more
224 cautious offspring (scototaxis), while predator-exposed fathers produced sons, but not daughters,
225 that were more risk prone (more active and exploratory in open field assays). Different effects of
226 maternal and paternal treatment on offspring could reflect the different proximate mechanisms
227 that mediate the transmission of cues from mothers versus fathers to offspring (e.g., eggs versus
228 sperm) and/or parent-of-origin effects (35). They might also reflect differences between mothers
229 and fathers in their ability to detect and/or respond to environmental conditions. From an
230 ultimate perspective, divergent maternal and paternal effects may have evolved in response to
231 sexual conflict, with mothers and fathers favoring different optimal offspring phenotypes (36).
232 There were non-additive interactions between the maternal and paternal environment on
233 some (survival, gene expression), but not all (scototaxis, open field behavior) offspring traits. In

234 particular, offspring of predator-exposed fathers had reduced survival against a live predator;
235 however, offspring of two predator-exposed parents did not have reduced survival, suggesting
236 that maternal predation exposure may mitigate the deleterious effects of paternal predation
237 exposure to some degree. Despite the fact that maternal cues seemingly over-rode the effect of
238 paternal cues on survival, we did not find evidence that maternal cues were necessarily more
239 dominant at the molecular level, as comparable numbers of genes were differentially expressed
240 in response to maternal versus paternal treatment. Moreover, the brain gene expression profile of
241 offspring of two predator-exposed parents did not more closely resemble the gene expression
242 profile of offspring of predator-exposed mothers. Instead, our results are more consistent with
243 the hypothesis that non-additive interactions between the environments experienced by mothers
244 and fathers produce a distinct neurogenomic profile. These interactive effects could arise due to
245 epigenetic mechanisms such as parent-of-origin effects (37) or because paternal cues via sperm
246 are mediated by maternal contribution to cytoplasm in the developing embryo (19).
247 In addition to interactions between the maternal and paternal environment, we found
248 strong evidence that sons and daughters differ in their phenotypic response to maternal and
249 paternal exposure to predation risk. These sex-specific patterns emerged in our study well before
250 offspring were reproductively mature, during a period in their life when males and females are
251 shoaling and still occupying similar habitats (27). Interestingly, these sex-specific patterns of
252 transgenerational plasticity did not seem to emerge along a consistent male-female divide (e.g.
253 sons attend to their father and daughters attend to their mother); instead, sons and daughters were
254 altered by paternal and maternal environments at a relatively similar magnitude, but in different
255 ways. These sex-specific effects may result from differences in sons and daughters in their
256 susceptibility to parental stress (38, 39) or may evolve due to parent-offspring conflict, in which

sons and daughters have different capacities to respond to or ignore information from fathers and mothers. It is also possible that sex-specific responses are adaptive for offspring, with differences organizing in early development to allow offspring to develop phenotypes that are better matched to the different environments they will encounter later in life. For example, it is possible that increased risk-prone behavior for sons, but not daughters, may be adaptive because high variance in male reproductive success favors males that adopt high risk, high reward behaviors to increase growth and access to resources under high predation pressure (29). Our study shows that maternal and paternal predation exposure can have fitness consequences for offspring (i.e., via survival) in the lab; work is needed in a more natural context in the field to assess whether these parental cues have adaptive or maladaptive consequences.

Interactions between the parental environment and offspring sex could be mediated via a variety of proximate mechanisms. The interactive effects in the gene expression data were not restricted to genes located on the nascent sex chromosomes (SI Appendix), but could arise from trans-acting mechanisms (e.g., regulation of genes on non-sex chromosomes by genes located on the sex chromosome (14)) or by sex-specific differences in epigenetic mechanisms. Genomic imprinting can allow favorable sex-specific traits to persist across generations by allowing offspring to only express alleles from one parent, such as the parent that has more reliable information about the environment offspring will encounter (e.g. the parent that does not disperse) (21, 40). Further, in bulls, Y-bearing and X-bearing spermatozoa have differentially expressed proteins, suggesting a mechanism by which fathers can transmit different information to sons versus daughters (41). Although mothers in many species can also transmit different information to sons and daughters (e.g., via placental function and gene expression (38, 39)), it is unclear if mothers can transmit different information to sons and daughters in externally

fertilizing species such as sticklebacks, in which mothers do not interact with their offspring post-fertilization.

In contrast to systems with internal fertilization (e.g., mammals), in this experiment we could completely isolate maternal versus paternal effects mediated via either eggs or sperm because there was no opportunity for parents to interact pre-fertilization or to influence offspring post-fertilization. This allowed us to control for mate choice, differential allocation due to partner quality, and differential allocation mediated via gestation and parental care (including the selective failure of stressed parents to provide care or successfully rear offspring). All of these can generate interactions between maternal phenotypes, paternal phenotypes, and offspring sex (38, 42-44) and obscure the ability to understand whether these sex-specific effects can arise via epigenetic changes to gametes. Here, we show that these distinct and interactive effects of maternal and paternal effects can be mediated via selective changes to information encoded in eggs and sperm. A fascinating direction for future work would be to consider how parenting and mate choice might influence these results by altering the magnitude and interactive nature of maternal and paternal effects, the extent to which parental effects selectively influence sons and daughters, and whether these parental effects have adaptive or maladaptive consequences.

In conclusion, we show here that both the sex of the parent and the sex of the offspring are important for predicting the ways in which offspring phenotypes are altered by parental experiences. We demonstrate that paternal cues mediated via sperm seem to be just as prominent as maternal cues mediated via eggs. However, these sex-specific patterns would have been masked if we had combined cues coming from mothers and fathers (i.e. compared offspring of two predator-exposed parents to a control) or failed to isolate effects emerging in sons versus daughters. These sex-specific effects might be favored when it is not adaptive for both sexes to

have the induced phenotype or when the same environment favors different phenotypes in males and females. Collectively, these results suggest that current theoretical and empirical work seeking to understand the evolution of transgenerational plasticity would benefit from considering the conditions which favor *sex-specific* patterns of transgenerational plasticity. Further, given broad interest in understanding the consequences of transgenerational plasticity for future generations and its potential to influence adaptive evolution, future work should consider how sex-specific effects in the first generation may alter the ways in which transgenerational effects persist for multiple generations in lineage-specific or sex-specific ways.

Methods

Housing conditions. Adult, freshwater threespined sticklebacks were collected from Putah Creek (CA, USA). This population has prickly sculpin (*Cottus asper*), which preys primarily on stickleback eggs, fry, and juveniles. Females were housed in six groups of n=10 fish per tank to mimic shoaling conditions in the wild. To simulate predation risk, we used a clay model sculpin (21cm long) to chase females for 90 seconds each day; unexposed treatment tanks were left undisturbed (similar to (45)). Gravid females were removed from tanks and stripped of their eggs for in-vitro fertilization. Mothers were chased between 16-44 days; longer exposure increased offspring length at 4.5 months, but the length of exposure did not significantly alter any other measured offspring traits (SI Appendix).

Males were housed singly to build nests. Once their nest was completed, predator-exposed males were chased by a model sculpin for 30 sec every other day for 11 days; control males were left undisturbed. We chased males both less frequently and for less time than females because males were exposed alone (to mimic breeding season behavior). The day after the last

326 exposure, males were euthanized to obtain sperm for *in-vitro* fertilization. Stickleback males
327 produce sperm in the beginning of the breeding season (46); thus, paternal cues mediated via
328 sperm in this experiment are likely due to modifications to mature sperm.
329 F1 offspring were generated using a split clutch design, resulting in: 1) offspring of
330 unexposed fathers and mothers (n=11 half-clutches), 2) offspring of exposed fathers and
331 unexposed mothers (n=11 half-clutches), 4) offspring of unexposed fathers and exposed mothers
332 (n=10 half-clutches), and 4) offspring of exposed fathers and mothers (n=10 half-clutches). By
333 artificially fertilizing the eggs and incubating the embryos using an air bubbler, we controlled for
334 possible pre-fertilization effects mediated by interactions between mothers and fathers (42, 44),
335 as well as the post-fertilization effects mediated by paternal care (47). Separate groups of
336 offspring were used for each assay described below (see SI Appendix for detailed methods and
337 statistical analysis).
338
339 ***Measuring survival under predation risk and ventilation rate***. At 3-5 months of age,
340 groups of n=4 offspring (one from each parental treatment) were exposed to a live sculpin
341 predator. One day prior to the predation assay, fish were weighed, measured, and individually
342 transferred to a 250ml opaque glass beaker containing 100mL of water. We measured opercular
343 beats 30 seconds after transferring to the beaker as a proxy for acute stress (29) and 30 minutes
344 after transferring to understand response to prolonged stress (n=100 fish per parental treatment
345 group). At the end of thirty minutes, all four fish were moved to the same holding tank until the
346 predation trial the following day. For the predation trial, sticklebacks were simultaneously
347 transferred into the sculpin's tank (n=4 different sculpin, each used once per day); the trial ended
348 two minutes after the first fish was captured by the sculpin. 14/100 trials did not result in any

successful captures and were excluded from further analysis. We euthanized the survivors of the predation assays and used a section of muscle tissue to sex a large portion of the survivors per the methods of Peichel, *et al.* (48).

Measuring risk taking behavior. When offspring were 4.5 months, we measured behavior in an open field before and after a simulated predator attack (as in (49)). Individuals were placed in an opaque refuge in the center of a circular arena divided into nine sections. After a three minute acclimation period, we removed the plug from the refuge, measured the latency for fish to emerge, and then measured the number of different (exploration) and total (activity) sections visited for three minutes after emergence. We then simulated a sculpin predator attack and measured the latency to resume movement after the simulated attack. Once the individual resumed movement, we again measured the number of different and total sections visited for three minutes. We weighed and measured the fish, euthanized it via decapitation, and preserved the body in ethanol for identification of sex (48). We assayed n=118 fish: n=12 females and n=18 males with control parents, n=15 females and n=16 males with predator-exposed fathers, n=13 females and n=14 males with predator-exposed mothers, and n=11 females and n=19 males with two predator-exposed parents.

Measuring anxiety/cautiousness. Scototaxis (light/dark preference) protocols have been developed to test anti-anxiety/cautious behavior in fish (30). Fish were placed in a clear cylinder in the center of a half-black, half-white tank. After a 5-minute acclimation period, we lifted the cylinder, and fish explored the tank for 15 minutes, during which we measured the latency to enter the white section, total time in the white section, and the number of times the fish moved

between the black/white sections. Principal components analysis (R package factoextra) was used to combine these behaviors into one principal component (eigenvalue 2.10, captured 70.1% of the variance in behaviors). We interpret greater activity (duration/visits) in the white portion of the tank as anti-anxiety/cautious behavior (30). We assayed n=162 fish: n=23 females and n=15 males with control parents, n=22 females and n=17 males with predator-exposed fathers, n=23 females and n=21 males with predator-exposed mothers, and n=24 females and n=17 males with two predator-exposed parents.

Measuring brain gene expression. We dissected whole brains from 4.5 month juvenile offspring (n=5 male and n=5 female offspring per treatment group) and preserved brains in RNAlater. We extracted RNA using Mackerey-Nagel NucleoSpin 96 kits and sent n=39 samples to the Genomic Sequencing and Analysis Facility at UT Austin for TagSeq library preparation and sequencing (one sample was of poor quality). To estimate differential expression, pairwise comparisons between the experimental conditions (offspring of predator-exposed mothers, offspring of predator-exposed fathers, offspring of predator-exposed mothers and fathers) relative to the control condition (offspring of unexposed parents) within each sex were made using edgeR (50). To call differential expression, we used a 'glm' approach and adjusted actual p-values via empirical FDR, where a null distribution of p-values was determined by permuting sample labels for 500 times for each tested contrast and a false discovery rate was estimated (51).

In a separate analysis, WGCNA was used to cluster genes into co-expressed gene modules (52, 53). To find modules significantly associated with treatment effects, we fitted a linear model (54) which blocked for clutch ID as random factor, along with main and interactive effects of sex, paternal treatment, and maternal treatment on module eigengenes. Eigengenes

395 which were significantly associated ($p < 0.05$) with either the main or interactive effects of sex,
396 paternal treatment, and maternal treatment were retained.
397
398 ***Animal welfare note.*** All methods were approved by Institutional Animal Care and Use
399 Committee of University of Illinois Urbana-Champaign (protocol ID 15077), including the use
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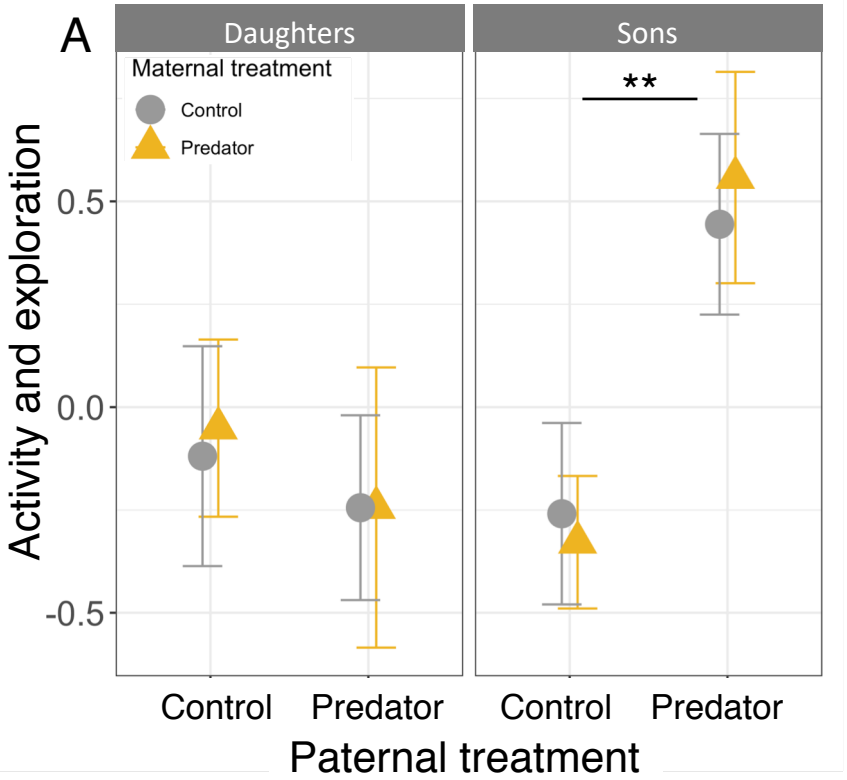
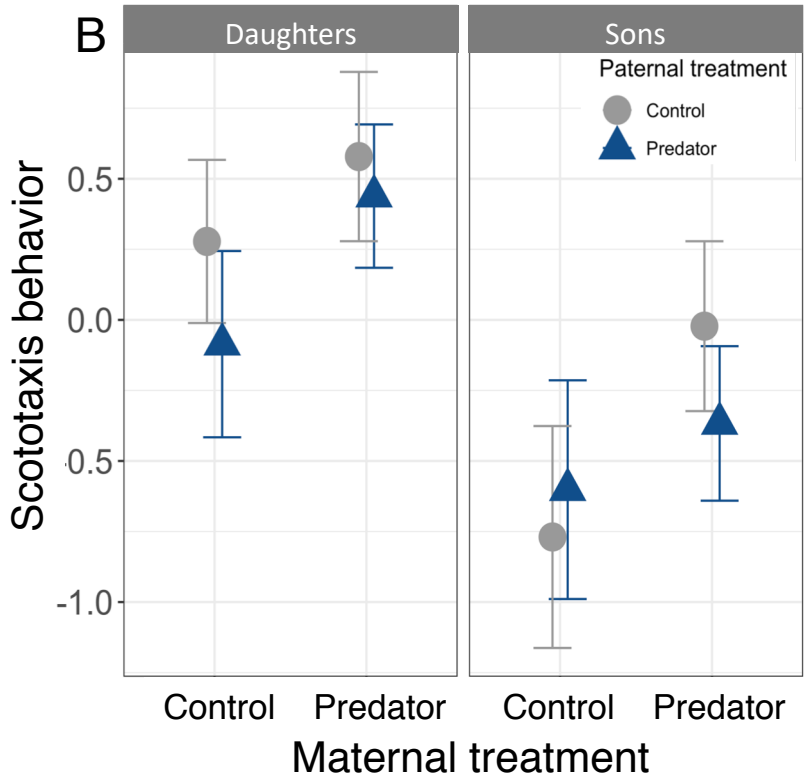
Figure Legends

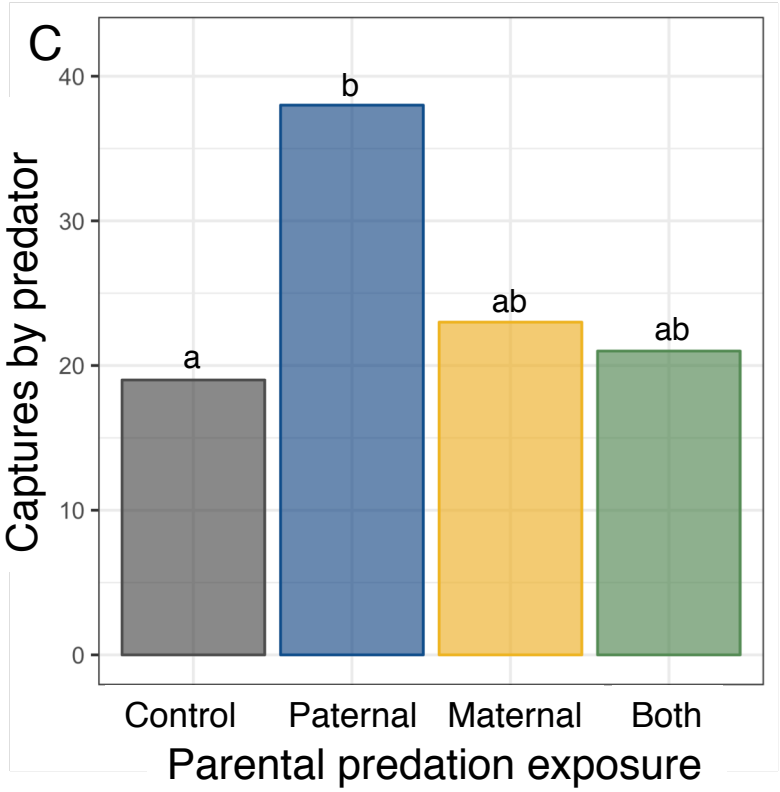
Figure 1: The effects of maternal and paternal treatment on offspring in an open field assay, scototaxis assay, and survival in the face of a live predator. A) Male offspring (right) of predator-exposed fathers were significantly more exploratory and active (PCA: higher values indicate more active and exploratory individuals; mean ± s.e.) compared to male offspring of control fathers; paternal treatment did not affect the exploratory behavior/activity of female offspring (left). The effect of paternal treatment did not depend on maternal treatment (control: grey; predator-exposed: yellow). N= 118 offspring. Stars indicate significant differences across treatment groups. B) Offspring of predator-exposed mothers were more cautious (PCA: high values indicate longer latency to enter the white area and spent less time in the white area; mean ± s.e.) compared to offspring of control mothers. Further, female offspring (left) were more cautious than male offspring (right). The effect of maternal treatment did not depend on paternal treatment (control: grey; predator-exposed: blue). N= 162 offspring. C) In live predation trials, juvenile offspring of predator-exposed fathers, but not two predator-exposed parents, were significantly more likely to be captured and consumed by the sculpin predator relative to offspring of control fathers. Letters indicate significant differences among treatment groups, determined by Tukey's HSD with parental treatment as a 4-level variable. N= 86 trials. Within each figure, data are plotted to facilitate visualization of the statistically significant interaction terms.

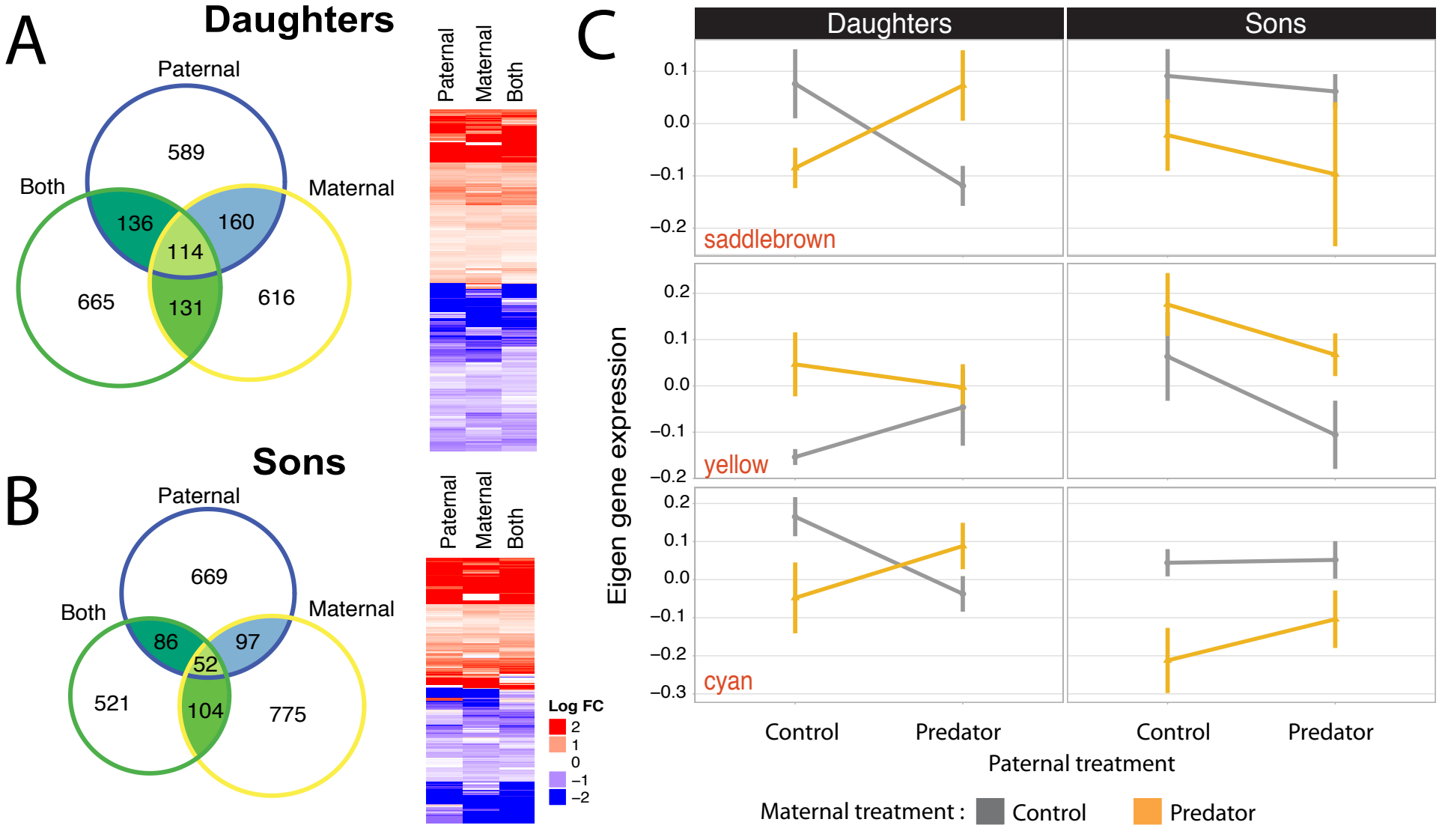
Figure 2: Differential gene and eigen-gene expression analysis. A-B) The three circles in the Venn diagram show the number of genes that were differentially expressed in the brain of offspring of unexposed parents relative to offspring of predator-exposed mothers ("maternal"),

predator-exposed fathers ("paternal"), or two predator-exposed parents ("both"), with daughters in (A) and sons in (B). Note that relatively few genes overlap between the different pairwise comparisons. The heatmaps show the direction of gene regulation (blue: downregulated; red: upregulated) of the differentially expressed genes that are shared among the three pairwise comparisons, with daughters and sons shown separately. C) The expression profiles of the four eigen-gene modules which were significantly affected by the three-way interaction among paternal treatment, maternal treatment and offspring sex (mean ± s.e.). N=39 offspring.


A
Daughters
Sons
Maternal treatment
Control
Predator
**
Activity and exploration
0.5
0.0
-0.5
Control
Predator
Control
Predator
Paternal treatment


B
Daughters
Sons
Paternal treatment
Control
Predator
Scototaxis behavior
0.5
0.0
0.5
-1.0
Control
Predator
Control
Predator
Maternal treatment


C
Captures by predator
40
30
20
10
0
a
b
ab
ab
Control
Paternal
Maternal
Both
Parental predation exposure


A
Daughters
Paternal
Both
Maternal
589
136
160
114
665
131
616
Paternal
Maternal
Both
B
Sons
Paternal
Both
Maternal
669
86
97
52
521
104
775
Paternal
Maternal
Both
Log FC
2
1
0
−1
−2
C
Daughters
Sons
saddlebrown
yellow
cyan
Eigen gene expression
0.1
0.0
−0.1
−0.2
0.2
0.1
0.0
−0.1
−0.2
0.2
0.1
0.0
−0.1
−0.2
−0.3
Control
Predator
Control
Predator
Paternal treatment
Maternal treatment :
Control
Predator