Sex-specific transgenerational plasticity in threespined sticklebacks

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15 **Introductory paragraph**

16 Sex-specific selection pressures can generate different phenotypic optima for males and
17 females in response to the current environment. Less widely appreciated is the possibility of sex-
18 specific transgenerational plasticity (TGP): mothers and fathers may exert different effects on
19 offspring traits and parental cues may persist selectively across generations via only daughters or
20 sons. Here, we demonstrate that maternal and paternal exposure to predation risk has largely
21 distinct effects on offspring behavior in threespined sticklebacks (*Gasterosteus aculeatus*), with
22 non-additive interactions between maternal and paternal effects on offspring survival and brain
23 gene expression profiles. Further, parental effects on offspring behavior and brain gene
24 expression profiles varied between male and female offspring, suggesting that mothers and
25 fathers activate different developmental programs in sons versus daughters. Altogether these
26 results demonstrate that sex- both of the parent and offspring- influences TGP patterns in ways
27 that may reflect the distinct life history trajectories of males and females.

28

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

Sex differences in life-history (e.g. mortality rate) or reproductive strategies can favor different optimal phenotypes in males and females [1]. Sex-specific patterns of within-generational plasticity have been documented in diverse taxa [2, 3, 4, 5]. While less explored, there also is evidence for sex-specific ***transgenerational*** plasticity (TGP; also referred to as intergenerational plasticity or environmental parental effects); 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 [6, 7, 8] and the influence of parental cues (whether mediated by the mother or the father) often depends on the sex of the offspring [9, 10, 11, 12, 13, 14, 15].

Recent theoretical models exploring the evolution of TGP assume that mothers transmit information about the current environment and offspring integrate maternal cues with environmental cues provided by genes and early-life experiences [16, 17, 18, 19]. However, these models largely do not consider that offspring simultaneously integrate information from mothers and fathers, such that the influence of maternal cues might depend on paternal cues (or vice versa) [7, 20, 21, 22]. Similarly, offspring might selectively respond to information from one parent over the other, a pattern that is largely underexplored, but likely if one parent is a more reliable source of environmental information (e.g. the same-sex parent, the non-dispersing parent). Interactions between maternal cues, paternal cues, and offspring sex are probably common given the prevalence of sex-specific developmental plasticity, and may be central to understanding the evolution of TGP.

Here, we evaluate the potential for interactions between maternal cues, paternal cues, and offspring sex in threespined sticklebacks (*Gasterosteus aculeatus*). Understanding the ways in which the maternal cues, paternal cues, and offspring sex interact during TGP could help clarify

54 evolutionary phenomena such as sexual conflict, parent-offspring conflict, and genomic
55 imprinting, which is thought to arise from sexual conflict over resource allocation to offspring.
56 For example, sexual conflict may cause mothers and fathers to favor different phenotypes in their
57 offspring, resulting in the evolution of mechanisms that allow mothers to manipulate the ways in
58 which fathers influence offspring (e.g. via cytoplasmic contributions [23]) or fathers to manipulate
59 the ways in which mothers influence offspring (e.g. via ejaculate composition [24]). Further,
60 nongenetic inheritance that functions in a sex-specific manner can resolve evolutionary conflicts
61 that occur when selection favors different phenotypes in sons and daughters [25] because male
62 versus female offspring may integrate parental information in ways that match their distinct life
63 history trajectories. One way that such sex-specific inheritance could operate is if cues from
64 mothers and fathers activate different developmental programs in daughters and sons.
65 Male and female sticklebacks are sexually dimorphic in several respects, such as habitat
66 use [26] and diet [27], and have a variety of male-specific reproductive traits that increase male
67 vulnerability to predation risk [28, 29]: male sticklebacks develop bright nuptial coloration, engage
68 in conspicuous territory defense and courtship behavior, and are the sole providers of paternal
69 care that is necessary for offspring survival [30]. These sex differences in behavior and life history
70 likely alter the predation regimes experienced by mothers versus fathers [31] and the optimal
71 phenotype for daughters versus sons in response to predation risk. We exposed adult male and
72 female sticklebacks to simulated predation risk prior to fertilization and used a fully factorial
73 design to generate offspring of control (unexposed) parents, offspring of predator-exposed
74 mothers, offspring of predator-exposed fathers, and offspring of predator-exposed mothers and
75 fathers. Because predation risk varies in both space and time, it is likely that there is a mix of
76 reproductively mature males and females who either have or have not recently experienced

77 predation risk within many natural populations. We reared sons and daughters under 'control'
78 conditions (i.e. in the absence of predation risk). We evaluated traits relevant to predator defense
79 and used brain gene expression data to gain insights into the underlying mechanisms.
80
81 **Results**
82 ***Sons, but not daughters, of predator-exposed fathers were more active under risk***
83 We compared maternal and paternal exposure to predation risk on the risk-taking
84 behavior of sons and daughters (n=118 offspring). We used an open field assay to measure
85 offspring activity/exploration and boldness under baseline conditions and after a simulated
86 predator attack. Offspring were significantly less active/exploratory after the simulated predator
87 attack compared to before (principal component analysis: higher values indicate more active and
88 explorative individuals; Table 1), confirming that offspring behaviorally responded to the
89 predator attack. There was a significant interaction between paternal treatment and offspring sex
90 on offspring activity/exploration (Table 1; Figure 1A). Specifically, sons of predator-exposed
91 fathers were significantly more active/exploratory compared to sons of control fathers (95% CI
92 in brackets here and below [-1.30, -0.20], p=0.01), but there was not a detectable effect of
93 paternal treatment on female offspring ([-0.40, 0.81], p=0.49). This suggests that sons were
94 especially responsive to paternal exposure to predation risk. Greater activity in response to
95 exposure to predation risk is consistent with higher risk-taking behavior observed in sticklebacks
96 from high predation populations compared to low predation populations [32].
97 We did not detect a significant effect of maternal or paternal treatment on boldness
98 (principal component analysis: higher values indicate less bold fish with an increased latency to
99 emerge from the shelter and to resume movement after the predator attack), although female

offspring were less bold than male offspring (Table 1). We found no evidence that standard length or body mass at 4.5 months were significantly influenced by maternal (SL [1.14, 1.99], p=0.67; mass [-0.03, 0.02], p=0.91) or paternal (SL [-1.77, 1.38], p=0.78; mass [-0.03, 0.03], p=0.96) exposure to predation risk. Standard length ([-1.24, 0.35], p=0.25) and mass ([-0.003, 0.01], p=0.20) also did not vary between male and female offspring, although larger fish were less active/exploratory and less bold (Table 1).

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 [33]. In order to determine whether a parent's experience with predation risk influences the anxiety-like behavior of their offspring [34], we conducted light-dark preference tests in a half-black/half-white tank (n=162 offspring). 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 ([-0.79, 0.32], p=0.44). Both female ([-1.27, -0.17], p=0.01) and smaller ([-0.10, -0.006], p=0.03) offspring showed more cautious behavior. We found no evidence of seasonal effects (experimental day [-0.004, 0.01], p=0.33).

Mothers mitigated the fitness costs of paternal exposure to predation risk

To understand if parents' experience with predation risk altered offspring survival in an encounter with a predator (reviewed in Sheriff, MacLeod [35]), we measured offspring survival

against live sculpin predators (n=100 trials) as well as response to an acute stressor (confinement stress, 30 seconds and 30 minutes after initial confinement, n=400 individuals per timepoint). There was a significant interaction between maternal and paternal treatment on offspring survival in live predation assays (generalized linear mixed effect model: Z_{335} = -1.98, 0.047). Specifically, offspring of predator-exposed fathers were more frequently captured by the predator compared to offspring of control parents, but this was not true for offspring of predator-exposed mothers or both a predator-exposed mother and father (Figure 1C). This suggests that was a strong fitness cost of having a predator-exposed father, but mothers seemed to mitigate those costs, perhaps by making their offspring more cautious (see above). Survivors of the successful predation trials were heavily female biased (93/148; Chi-squared: χ^2=9.76, p=0.002), suggesting that males are generally more vulnerable to predation risk. The sex-bias was not significantly different across treatment groups (χ^2=3.03, p=0.39). We found no effect of size on how frequently the stickleback were captured by the predator (Z_{335} = 1.56, 0.11).

We found no significant difference between stress-induced respiration rates after initial confinement or after 30 minutes of confinement (95% CI [-2.77, 3.16], p=0.90). We did not detect a significant effect of maternal ([-4.79, 12.44], p=0.36) or paternal treatment ([-16.91, 4.05], p=0.20) on stress-induced respiration, although larger fish tended to have lower stress-induced respiration compared to smaller fish ([-2.24, 0.14], p=0.08). For the portion of offspring where sex was known, we found non-significant interactions between offspring sex and paternal ([-17.67, 14.83], p=0.89) or maternal treatment ([-23.89, 8.77], p=0.37), although males tended to have higher opercular beats than females (main effect of sex [-1.48, 25.92], p=0.08). Individuals with lower opercular beat rate at initial confinement (Z_{336}= -1.92, p=0.05) and after

30 minutes of confinement (Z_{336}= -1.75, p=0.08) tended to be more likely to be captured by the predator.

Distinct and nonadditive effects of maternal and paternal treatment on offspring brain gene expression

The results described above suggest that predation risk experienced by mothers versus 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 with a predator-exposed mother, a predator-exposed father, and two predator-exposed parents in male and female offspring (n=39 individuals). In terms of the number of genes, the effects of maternal and paternal treatment on brain gene expression were approximately equivalent in magnitude, and the genes 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 effects 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-

167 exposed mothers and fathers, there is a core set of genes that is activated in offspring brains in
168 response to either maternal or paternal exposure to predation risk.
169
170 ***Maternal and paternal exposure to predation risk interacted with offspring sex to influence***
171 ***offspring brain gene expression***
172 The behavioral data suggest that sons and daughters respond to parental experience with
173 predation risk differently, with sons, but not daughters, increasing activity/exploration in
174 response to paternal experience with predation risk. One way that such sex-specific inheritance
175 could arise is if cues from one parent (e.g. fathers) activate a particular developmental program
176 in one offspring sex but not the other (e.g. in sons but not daughters).
177 To test this hypothesis, we used WGCNA to identify clusters ("modules") of genes with
178 coordinated expression patterns. This procedure reduced the dimensionality of the transcriptomic
179 dataset, which allowed us to explore the potential for interactive effects of maternal treatment,
180 paternal treatment and offspring sex on modules of genes with correlated expression patterns.
181 WGCNA identified 23 informative modules in the dataset. The expression of eight of the 23
182 modules was significantly affected by at least one of the factors in the model: three modules
183 were significantly affected by maternal treatment, two were significantly affected by the two-
184 way interaction between maternal and paternal treatment, and three were significantly affected
185 by the three-way interaction between paternal treatment, maternal treatment and offspring sex
186 (shown in Figure 2C). For example, the module "saddle brown" comprises 48 co-expressed
187 genes (largely enriched for developmental processes) whose expression was influenced by the
188 three way interaction between maternal treatment, paternal treatment and offspring sex.
189 Specifically, daughters of a predator-exposed mother or father 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

Transgenerational plasticity can allow environmental information to be delivered to offspring earlier and with potentially lower costs to offspring than developmental plasticity, which may allow offspring to develop traits during early development that help them cope with environmental change [36, 37]. Unlike genetic inheritance, TGP can potentially be fine-tuned to the precise environment that both parents and offspring will encounter [25], including the different environments experienced by males and females because of sex differences in life history and reproductive strategies. The results reported here draw attention to the importance of sex-specific TGP: offspring phenotypes varied depending on whether predation risk had been experienced by their mother or their father, and a parent's experience with predation risk produced different phenotypes in their sons compared to their daughters.

We found that maternal and paternal exposure to the same environmental factor (predation risk) generated largely distinct effects in offspring: predator-exposed mothers produced more cautious offspring (scototaxis), while predator-exposed fathers produced sons, but not daughters, that were more active under risk (more active and exploratory in open field assays). There were also non-additive interactions between maternal and paternal effects on

some (survival, gene expression), but not all (scototaxis, open field behavior) offspring traits. In particular, offspring of predator-exposed fathers had reduced survival against a live predator; however, offspring of two predator-exposed parents did not have reduced survival, suggesting that maternal predation exposure may mitigate the deleterious effects of paternal predation exposure to some degree. Despite the fact that maternal effects seemingly over-rode paternal effects on survival, we did not find evidence that maternal effects were necessarily more dominant at the molecular level, as comparable numbers of genes were differentially expressed in response to maternal versus paternal treatment. Moreover, the brain gene expression profile of offspring of two predator-exposed parents did not more closely resemble the gene expression profile of offspring of predator-exposed mothers. Instead, our results are more consistent with the hypothesis that non-additive interactions between the environments experienced by mothers and fathers produce a distinct neurogenomic profile.

In addition to interactions between maternal and paternal effects, we found strong evidence that sons and daughters differ in their phenotypic response to maternal and paternal exposure to predation risk. These sex-specific patterns emerged in our study well before offspring were reproductively mature, during a period in their life when males and females are shoaling and still occupying similar habitats [30]. Interestingly, these sex-specific patterns of transgenerational plasticity did not seem to emerge along a consistent male-female divide (e.g. sons attend to their father and daughters attend to their mother); instead, sons and daughters were altered by paternal and maternal environments at a relatively similar magnitude, but in different ways. These sex-specific effects may result from differences in sons and daughters in their susceptibility to parental stress [38, 39] and/or may be adaptive for offspring, with differences originating 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 activity under risk 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 [32]. 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 effects have adaptive or maladaptive consequences.

Whether the fitness interests of mothers, fathers, and offspring align or conflict has important implications for understanding how and why sex-specific TGP evolves. On the one hand, sex-specific TGP may arise because mothers and fathers favor different optimal offspring phenotypes [40], and/or sons and daughters have different capacities to respond to or ignore information from fathers and mothers. If this is the case, TGP may evolve at the interface between sexual conflict and parent-offspring conflict, with paternal strategies, maternal strategies, and offspring counter-adaptations all ultimately dictating offspring phenotypes. On the other hand, parents' and offspring fitness interests in the face of predation risk may be aligned; if this is the case, then sex-specific plasticity may arise because mothers and fathers experience their environment in different ways and/or because the same parental environment favors different phenotypes in sons and daughters.

Interactions between maternal effects, paternal effects, and offspring sex could be mediated via a variety of proximate mechanisms. The distinct effects of maternal and paternal experiences could reflect different proximate mechanisms that mediate the transmission of cues from mothers versus fathers to offspring (e.g., eggs versus sperm) as well as the ways in which mothers and fathers were exposed to risk. Both distinct and interactive effects could also be

mediated by epigenetic mechanisms such as parent-of-origin effects [41, 42] or interactions between maternal and paternal contributions (e.g. egg cytoplasm altering the effect of sperm small RNAs) during early development [23, 24]. Differences between sons and daughters in how they respond to parental information could be mediated via trans-acting mechanisms (e.g., regulation of genes on non-sex chromosomes by genes located on the sex chromosome [12]), sex-specific differences in epigenetic mechanisms, or genomic imprinting [25, 43]. 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 [44]. 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. Future work exploring these proximate mechanisms would be fascinating for understanding the extent to which variation in parental effects is due to changes in the information encoded by parents or changes in offspring responsiveness to parental information.

Because parents can differentially allocate based on their partner's phenotype or environmental conditions experienced by their partner [21, 22, 45], in most systems it is difficult to isolate the effects of direct parental exposure to an environmental cue from environmental cues that parents indirectly detect from their mate (e.g. predator-naïve fathers provide less care to offspring of predator-exposed mothers) [21, 22, 45]. This makes it difficult to understand whether paternal effects can be mediated via sperm alone, or to determine the influence of paternal effects in isolation of maternal effects. In this experiment, we were able to completely isolate paternal effects mediated via sperm because there was no opportunity for parents to interact pre-

fertilization or to influence offspring post-fertilization. Although out results suggest that distinct and interactive effects of maternal and paternal effects can be mediated via selective changes to information encoded in eggs and sperm alone, a fascinating direction for future work would be to consider how parental care and mate choice might ameliorate or magnify the sex-specific effects observed here.

In conclusion, we show 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. Consequently, 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 and/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. The parental generation was maintained on a summer

photoperiod schedule (16 L : 8D) at 20° ± 1°C and fed ad libitum daily with a mix of frozen bloodworms (*Chironomus* spp.), brine shrimp (Artemia spp.) and Mysis shrimp.

To simulate natural conditions on the breeding grounds, where males defend nesting territories while females shoal together, we used different procedures for exposing mothers and fathers to predation risk. Mothers were housed in six groups of n=10 fish per tank to mimic shoaling conditions in the wild. To simulate predation risk, we randomly assigned three tanks to a predator-exposed treatment and we used a clay model sculpin (21cm long) to chase females for 90 seconds each day; the three unexposed treatment tanks were left undisturbed (similar to Dellinger, Zhang [46]). Females remained in the group tanks until they become gravid, at which time they were removed from the tank and stripped of their eggs to be used 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 (Supplementary Material).

Fathers were kept singly in 26.5L tanks (36L x 33W x 24H cm), visually isolated from the other males' tanks with opaque partitions. Each tank contained two plastic plants, a sandbox, a clay pot, and algae to encourage nest building. 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. A separate experiment confirmed that the results reported below were not produced when fathers were chased with a net (unpublished data), suggesting that changes in offspring traits are specific to predation risk and not a byproduct of, for instance, differences in activity levels due to chasing. The day after the last exposure, males were euthanized to obtain sperm for *in-vitro* fertilization. While female sticklebacks produce eggs throughout the breeding season, stickleback males produce sperm in the beginning of the

breeding season [47]; thus, paternal cues mediated via sperm in this experiment are likely due to modifications to mature sperm. Although mothers and fathers were exposed to risk differently, previous studies in this population suggest that exposure to predation risk for individuals who are isolated versus in groups have largely similar consequences: another experiment comparing post-fertilization paternal cues of predation risk with early life cues of predation risk found largely overlapping effects of paternal and personal experience with predation risk, despite the fact that fathers were exposed alone and offspring were exposed in family groups [48].

F1 offspring were generated via *in vitro* fertilization using a split clutch design. Each female's clutch was split and fertilized with sperm from both a predator-unexposed and predator exposed male, while each male's sperm was split and used to fertilized eggs from a predator-unexposed and predator exposed female, ultimately resulting in 42 clutches of half-siblings (some half clutches failed to fertilize or develop). This factorial design resulted in four different types of offspring: 1) offspring of unexposed fathers and mothers (n=11 half-clutches), 2) offspring of exposed fathers and unexposed mothers (n=11 half-clutches), 3) offspring of unexposed fathers and exposed mothers (n=10 half-clutches), and 4) offspring of exposed fathers and mothers (n=10 half-clutches). We incubated fertilized eggs in a cup with a mesh bottom placed above an air bubbler and fry were reared in 37.9 L tanks (53L x 33W x 24H cm). By artificially fertilizing and incubating the eggs, we controlled for possible pre-fertilization effects mediated by interactions between mothers and fathers [22, 45], as well as the post-fertilization effects mediated by paternal care [49]. Further, by artificially fertilizing and incubating the eggs, our experimental design controlled for the possibility that stressed parents might be less likely to successfully mate or parent offspring.

At 2.5 months, we split the five largest clutches in each of the four treatments. We gently caught individual fish in a clear bottomed cup and put 10 fish each into two 26.5L tanks. Fish in one of those 26.5L tanks were used for the behavioral assays and gene expression (see below). In the remaining clutches, 20 fish were caught and immediately returned to their tank. Offspring were switched to a winter light schedule (8 L: 16 D) prior to the predation trials, open field assays, or brain collection, but resumed a summer light schedule prior to the scototaxis assays, which were conducted when offspring were reproductively mature. Separate groups of offspring were used for each assay described below.

Measuring survival under predation risk and ventilation rate. At 3-5 months of age (mean: 20.6mm standard length ± 2.1 mm s.d), groups of n=4 offspring (one from each parental treatment) were exposed to a live sculpin predator. One day prior to the predation assay, one fish from each of the four parental treatments was gently caught from their home tank, weighed, and measured; fish within a trial were size matched as much as possible (mean pairwise standard length (SL) difference among stickleback per trial: 2.06mm ± 0.90 mm s.d). We gave each fish one mark with blue, yellow, orange, or red elastomer dye (Northwest Marine Technologies) on the side of their body. To control for potential correlations between color and survival, the color was rotated among trials such that all treatments received each color for one-fourth of the trials (captures did not vary by color: Chi-squared test, χ^2=3.50, p=0.32). After marking, each fish was transferred to a 250ml glass beaker containing 100mL of water, with opaque sides to isolate the fish. We measured opercular beats 30 seconds after transferring to the beaker as a proxy for acute stress [32] and 30 minutes after transferring to understand response to prolonged stress (n=100 fish per parental treatment group). At the end of thirty minutes, all four fish were moved

373 to the same 9.5L holding tank (32 x 21 cm and 19 cm high) until the predation trial the following
374 day.

375 Sculpin used as predators in this experiment (n=4) were housed individually in 26.5L
376 tanks (36L x 33W x 24H cm) with a bubbler, plants, and a clay pot for shelter. Each sculpin was
377 used for a maximum of one trial per day. One hour prior to the beginning of the trial, all bubblers
378 and plants were removed from the sculpin's tank and water was drained to the halfway point.
379 Immediately prior to the trial, the sticklebacks were gently netted from their holding tank, placed
380 in water in individual cups, and simultaneously transferred into the sculpin's tank as far away
381 from the sculpin as possible. The trial commenced as soon as all four fish were in the testing tank
382 and ended two minutes after the first fish was captured by the sculpin. We left the stickleback in
383 the tank for up to three hours and recorded the identity of the survivors. Of the 100 trials that
384 were conducted, 14 did not result in any successful captures and were therefore excluded from
385 further analysis of survival data. We euthanized the survivors of the predation assays and used a
386 section of muscle tissue to sex a large portion of the survivors (n=157 fish from 67 trials; tissue
387 samples were not collected for the first n= 22 trials) with a male-specific genetic marker per the
388 methods of Peichel, Ross [50].
389
390 ***Measuring behavior under predation risk.*** When offspring were 4.5 months, we
391 measured behavior in an open field before and after a simulated predator attack (as in Bensky,
392 Paitz [51]). Individuals were placed in an opaque refuge in the center of a circular arena (150cm
393 diameter) divided into eight peripheral sections with a circular section in the middle. After a
394 three minute acclimation period, we removed the plug from the refuge, measured the latency for
395 fish to emerge, and then measured the number of different (exploration) and total (activity)

sections visited for three minutes after emergence. Fish that did not emerge from the refuge after 10 minutes were gently released from the refuge; while offspring who emerged naturally were more active/exploratory than fish who were released (generalized linear model with binomial distribution (emerged or released), with activity/exploration as a fixed effect: Z_{234}=-3.68, $p<0.001$), controlling for emergence time did not alter the significance of the results reported below.

After the 3min period, we simulated a sculpin predator attack. This attack elicited freezing behaviour from the fish; we 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. If the fish remained frozen for greater than five minutes (n=20 fish), we ended the trial and considered activity and exploration after the simulated predation attack to be zero. Statistics were conducted on all the data, but results remain significant when these fish are omitted. We weighed and measured the fish, euthanized it via decapitation, and preserved the body in ethanol for identification of sex [50]. 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 [33]. When offspring were between 9-13 months old, offspring were gently caught with a cup from their home tank and placed in a clear cylinder (10.5cm diameter) in the center of a half-black, half-white tank (51L x 28W x 19H cm, coated on the inside and outside with matte contact paper), filled halfway with water. 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. The orientation of the tank was rotated between trials, and water was completely changed between each trial. On average, fish spent less time in the white portion of the tank than the black portion (mean ± s.e.: 208.7 ± 18.8 sec out of a 900 sec trial). We interpret greater activity (duration/visits) in the white portion of the tank as anti-anxiety/less cautious behavior [33]. After the 15-minute testing period, we removed the fish from the tank, recorded mass and standard length, euthanized the fish in an overdose of MS-222, and confirmed sex via examination of the gonads. 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 from each of the four parental treatment groups. We sampled n=2 offspring per family per treatment group (with 1 female and 1 male for most families), for n=5 male offspring and n=5 female offspring brains per parental treatment group. Offspring were captured from their home tank between 1100-1600hrs and immediately sacrificed. Brains were preserved in RNAlater, stored at 4°C overnight, and transferred to -80°C until RNA extraction. We extracted RNA using Macherey-Nagel NucleoSpin 96 kits, confirmed quality of samples via Bioanalyzer, normalized the concentration of the samples, 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).

Statistical Analysis.

Predation assays. We used generalized linear mixed models (GLMM) with a binomial distribution (R package lme4 [52]) to analyze differences in survival during the predation assay. We included fixed effects of maternal treatment, paternal treatment, and standard length, with random effects of maternal identity, paternal identity, sculpin identity, and test group. We tested for interactions between the fixed effects and removed all non-significant interactions.

Stress-induced respiration. Because we found evidence of heteroskedasticity in our data, we used MCMC generalized linear mixed models (R package MCMCglmm [53]) using a weak prior on the variance (V=1, nu=0.002). We ran models for 200,000 iterations, with a burn-in of 3000 iterations, thin = 3, and Gaussian distributions (and used these same parameters in MCMC GLMM models described in other sections below). To analyze differences in stress-induced respiration (breaths/minute), we included fixed effects of maternal treatment, paternal treatment, time period (30s or 30min after being placed in beaker), and standard length to control for differences in size. We also included random effects of individual identity nested within both maternal and paternal identity, to control for repeated measures on the same individual. We tested for interactions between fixed effects and removed all non-significant interactions. We removed one extremely low outlier in the opercular beat dataset. To determine if offspring sex predicted opercular beat rate, we reran the model above on the portion of offspring where sex was known (the survivors of the predation assays, n=157 offspring); we included all fixed and random effects stated above, plus an additional fixed effect of offspring sex. To determine if opercular beat rate predicted survival in the predation assays, we used GLMMs with a binomial distribution with survival as the dependent variable, opercular beat rate (square-root transformed)

as a fixed effect, and random effects of maternal identity, paternal identity, sculpin identity, and test group. We ran separate models for opercular beat rate at 30 seconds and 30 minutes.

Behavioral assays. We used principal components analysis (R package factoextra) to combine exploration (number of different sections visited) and activity (number of sections visited) into one principal component. Behaviors were scaled and centered, and we included activity and exploration before and after the simulated predator attack (two data points per individual). We extracted an eigenvalue of 1.79 that captured 89.3% of the variance in these two behaviors; positive values indicate more active and exploratory individuals. We also used a separate principal components analysis to combine latency to emerge from the shelter and latency to resume movement after the simulated predator attack, as these behaviors were correlated (Spearman rank correlation: $\rho=0.19$, $p=0.048$). We scaled and centered the behaviors, and extracted one principal component with an eigenvalue of 1.34 that captured 66.9% of the variance in these two behaviors; positive values indicate less bold individuals who took longer to emerge from the shelter and to resume movement after the simulated predator attack). We ran the second PCA independently of the first PCA because we had two data points per individual for activity and exploratory behavior (before and after the simulated predator attack) and only one data point per individual for latency to emerge and to resume movement.

To understand how parental exposure to predation risk altered behavior, length, and body mass, we used MCMC GLMMs with a Gaussian distribution. For activity/exploration, we included fixed effects of maternal treatment, paternal treatment, individual sex, standard length, and observation period (before or after the simulated predator attack), as well as random effects of ID nested within mother and father identity (separately) and observer identity. We combined

activity/exploration before and after the simulated predator attack into one model (two datapoints per individual) because we found no evidence of an interaction between maternal or paternal treatment with observation period. For emergence/freezing behavior we included fixed effects of maternal treatment, paternal treatment, individual sex, and standard length as well as random effects of mother identity, father identity, and observer identity. Finally, for models testing length and mass, we included fixed effects of maternal treatment, paternal treatment, individual sex, and random effects of maternal and paternal identity. We added an additional fixed effect of age (days since hatching) to the model testing length and an additional fixed effect of length for the model testing mass. We removed one outlier for the length dataset and one different outlier for the mass dataset. For all models, we tested for possible interactions between paternal treatment, maternal treatment, and offspring sex; we retained significant interactions.

Scototaxis. We used principal components analysis (R package factoextra) to summarize latency to enter the white section of the tank, the total amount of time the fish spent in the white portion of the tank, and the number of times the fish switched between the black and white side of the tank. For individuals that never entered the white side of the tank (n=30 of 162 individuals), we recorded latency to enter as 900 seconds, which was the duration of the trial. Behaviors were scaled and centered and we extracted one principal component with an eigenvalue of 2.10 that captured 70.1% of the variance for behavior in the scototaxis trials; positive values were a measure of increased cautious behavior with a higher latency to enter the white section, less total time in the white section, and fewer instances of switching between the black and white sections. We then used this principal component as the dependent variable in the MCMC GLMMs, with fixed effects of maternal treatment, paternal treatment, sex, standard

length, and day, to control for any potential season effects. We also included random effects of maternal and paternal identity, as well as observer identity. We tested for interactions between these fixed effects and removed all non-significant interactions.

TagSeq informatics. FASTQC was used to assess the quality of the reads. Tag-seq produced an average of ~7 million reads per sample. We aligned reads to the *Gasterosteus aculeatus* reference genome (the repeat masked reference genome, Ensembl release 92), using STAR (2.5.3) [54]. Reads were assigned to features according to the Ensembl release 92 gene annotation file (http://ftp.ensembl.org/pub/release-92/gtf/gasterosteus_aculeatus/). HTSeq-Count was used to count reads mapped to gene features using stickleback genome annotation. Multimapped reads or reads mapped to non-genic location were excluded from the analysis.

Differential gene expression. Two samples were excluded based on high variability on multidimensional scaling (MDS) plots. We included genes with at least 0.1 cpm in 5 samples. To estimate differential expression, pairwise comparisons between control and each treatment group (offspring with just a predator-exposed mother, with just a predator-exposed father, or two predator-exposed parents) within each sex using edgeR. Count data were TMM (trimmed mean of M-values) normalized and we used a 'glm' approach to call differential expression between treatment groups. We 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 [55].

Co-expression network analysis. To build an unsigned weighted co-expression network,

we excluded genes with non-zero variances. Further, we excluded genes that had zero counts in at least 80% of the samples. The input counts were voom transformed using voom functionality (R package limma) and then pairwise gene-gene correlations were estimated using Pearson correlation. Based on scale free topology criterion, spurious correlations were removed by estimating appropriate soft threshold. A soft threshold of 10 with almost 90% R square was used to filter low correlations and to build adjacency (A) and topological overlap (TOM) matrices. Then using hierarchical clustering, we built a dendrogram of all genes based on TOM. This tree was cut using dynamic tree cut method as implemented in WGCNA [56, 57] with deepSplit = 4 and minimum cluster size of 30 genes. Modules were further merged based on their similarity and their eigengenes values were saved for downstream analysis. Finally, genes with > 0.5 correlations with module eigengenes were retained as modules members.

To find modules significantly associated with treatment effects, we fitted a linear model which blocked for clutch ID as a random factor, along with main and interactive effects of sex, paternal treatment, and maternal treatment on module eigengenes using lmer test function in lmerTest package in R [58]. We used clutch ID for this analysis, rather than maternal and paternal identity separately, because we selected offspring from a subset of clutches and few of the clutch shared mothers or fathers. Eigengenes which were significantly associated ($p < 0.05$) with either the main or interactive effects of sex, paternal treatment, and maternal treatment were retained.

Animal welfare note. All methods were approved by Institutional Animal Care and Use Committee of University of Illinois Urbana-Champaign (protocol ID 15077), including the use of live predators.

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764 **Table 1:** Results of general linear mixed models (MCMCglmm) testing predictors of
765 exploration/activity (higher values indicate more active and exploratory individuals) and
766 boldness (higher values indicate less bold individuals, who took longer to emerge from the
767 shelter and resume movement after the simulated predator attack). We tested fixed effects of
768 maternal and paternal exposure to predation risk, sex, and standard length, with random effects
769 of maternal and paternal identity. Additionally, we included observation period (before or after
770 simulated predator attack) for activity/exploration, as well as random effects of ID nested within
771 maternal and paternal identity. Non-significant interaction terms were removed.

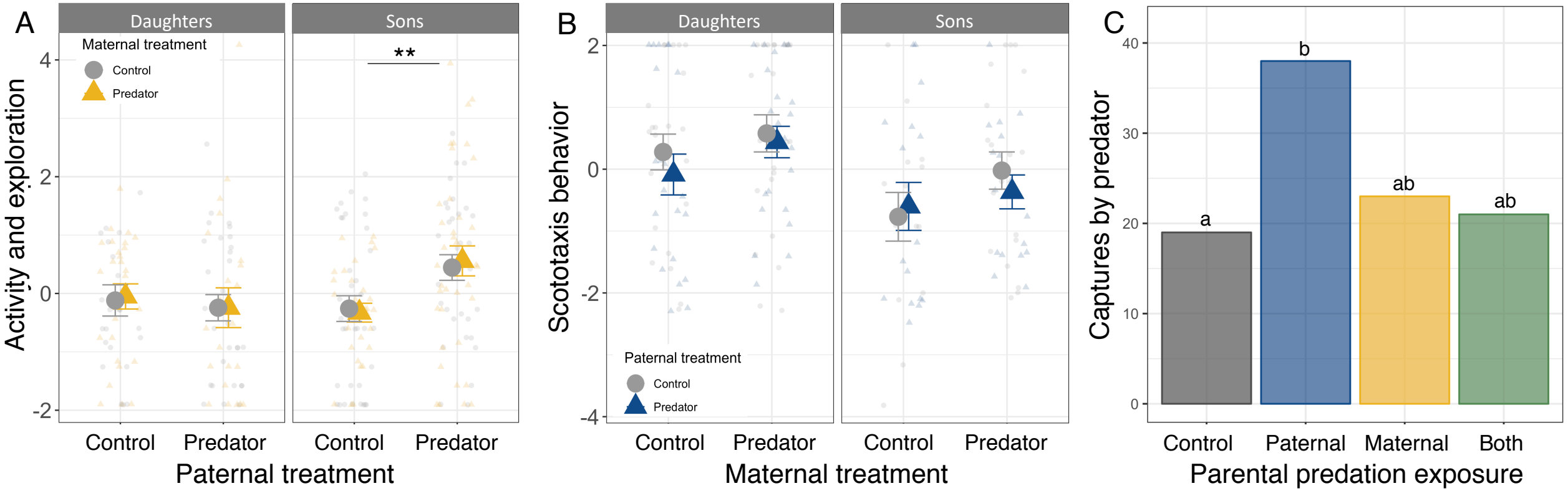
772
773
774

	Activity and exploration		
	Mean	*95% CI (L, U)*	*P*
Observation period	-0.97	-1.24, -0.70	**<0.001**
Maternal treatment	0.14	-0.27, 0.54	0.48
Paternal treatment	-0.20	-0.81, 0.42	0.52
Sex	-0.25	-0.70, 0.22	0.29
Standard length	-0.12	-0.18, -0.05	**<0.001**
Paternal treatment * sex	0.91	0.25, 1.54	**0.005**
	Latency to emerge and resume movement		
	Mean	*95% CI (L, U)*	*P*
Maternal treatment	0.27	-0.21, 0.73	0.25
Paternal treatment	-0.29	-0.82, 0.25	0.91
Sex	-0.45	-0.87, -0.06	**0.03**
Standard length	0.11	0.03, 0.19	**0.008**

775 **Figure Legends**

776 **Figure 1**: The effects of maternal and paternal treatment on offspring in an open field assay,
777 scototaxis assay, and survival in the face of a live predator. A) Male offspring (right) of predator-
778 exposed fathers were significantly more exploratory and active (PCA: higher values indicate
779 more active and exploratory individuals; mean ± s.e.) compared to male offspring of control
780 fathers; paternal treatment did not affect the exploratory behavior/activity of female offspring
781 (left). The effect of paternal treatment did not depend on maternal treatment (control: grey;
782 predator-exposed: yellow). N= 118 offspring. Stars indicate significant differences across
783 treatment groups. B) Offspring of predator-exposed mothers were more cautious (PCA: high
784 values indicate longer latency to enter the white area and spent less time in the white area; mean
785 ± s.e.) compared to offspring of control mothers. Further, female offspring (left) were more
786 cautious than male offspring (right). The effect of maternal treatment did not depend on paternal
787 treatment (control: grey; predator-exposed: blue). N= 162 offspring. C) In live predation trials,
788 juvenile offspring of predator-exposed fathers, but not two predator-exposed parents, were
789 significantly more likely to be captured and consumed by the sculpin predator relative to
790 offspring of control fathers. Letters indicate significant differences among treatment groups,
791 determined by Tukey's HSD with parental treatment as a 4-level variable. N= 86 trials. Within
792 each figure, data are plotted to facilitate visualization of the statistically significant interaction
793 terms and individual data points are shown for A and B.
794
795 **Figure 2**: Differential gene and eigen-gene expression analysis. A-B) The three circles in the
796 Venn diagram show the number of genes that were differentially expressed in the brain of
797 offspring of unexposed parents relative to offspring of predator-exposed mothers ("maternal"),

798 predator-exposed fathers ("paternal"), or two predator-exposed parents ("both"), with daughters
799 in (A) and sons in (B). Note that relatively few genes overlap between the different pairwise
800 comparisons. The heatmaps show the direction of gene regulation (blue: downregulated; red:
801 upregulated) of the differentially expressed genes that are shared among the three pairwise
802 comparisons, with daughters and sons shown separately. C) The expression profiles of the four
803 eigen-gene modules which were significantly affected by the three-way interaction among
804 paternal treatment, maternal treatment and offspring sex (mean ± s.e.). N=39 offspring.
805


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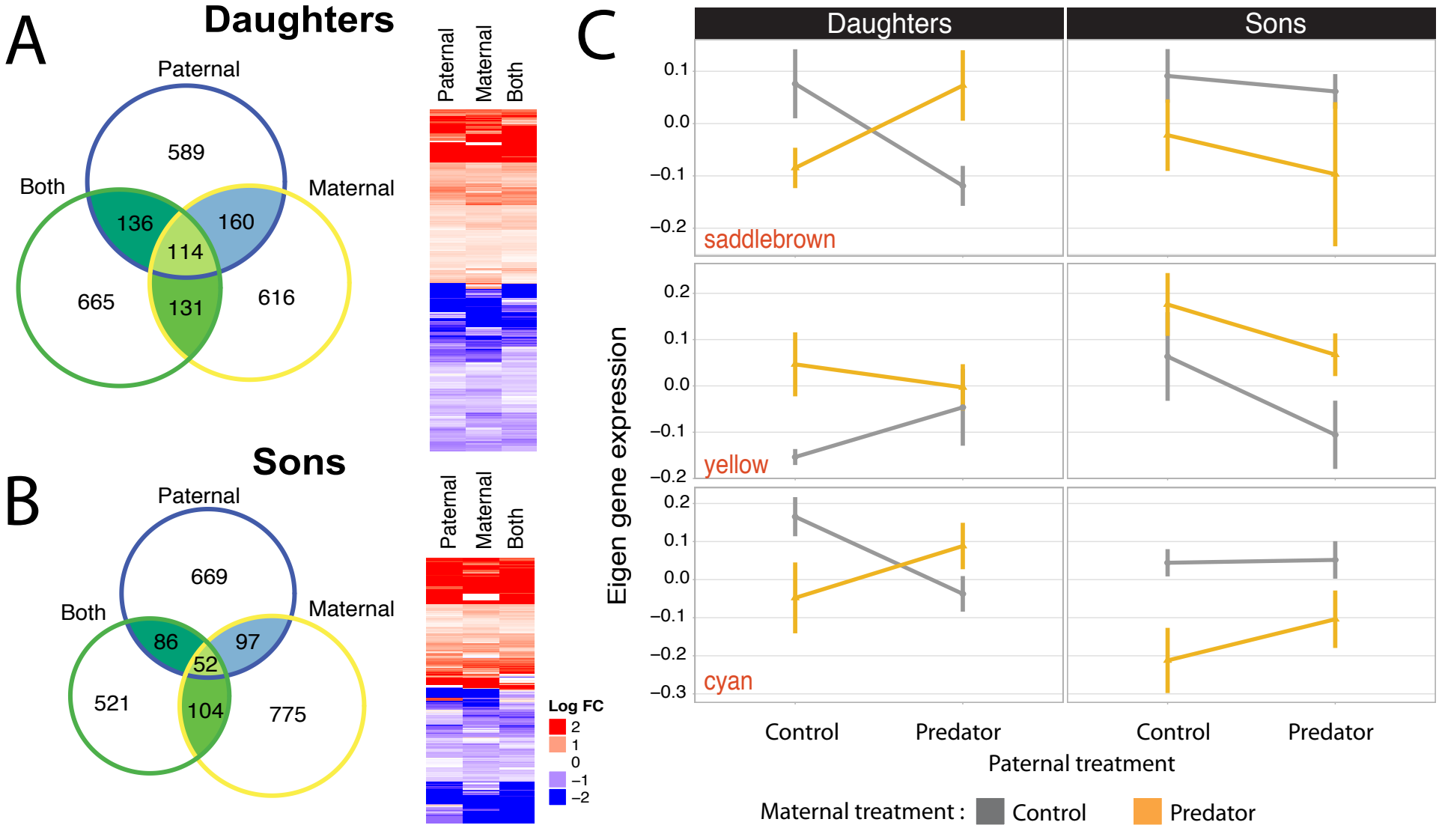
A
Daughters
Paternal
Both
Maternal
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136
160
114
665
131
616
Paternal
Maternal
Both
B
Sons
Paternal
Both
Maternal
669
86
97
52
521
104
775
Paternal
Maternal
Both
Log FC
2
1
0
−1
−2
C
Daughters
Sons
saddlebrown
yellow
cyan
Eigen gene expression
0.1
0.0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