Grandpaternal effects are lineage- and sex-specific in threespined sticklebacks

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Abstract

Transgenerational plasticity (TGP) occurs when the environment encountered by one generation (F0) alters the phenotypes of one or more future generations (e.g. F1 and F2). Selective inheritance of ancestral environments, via specific lineages or to only male or female descendants, may be adaptive if it allows past generations to fine-tune the phenotypes of future generations in response to sex-specific life history strategies. Here, we reared F1 offspring of unexposed and predator-exposed threespined stickleback (*Gasterosteus aculeatus*) fathers under 'control' conditions and generated F2s with a predator-exposed maternal and/or paternal grandfather. Grandpaternal effects were both sex and lineage-specific: female F2s were heavier and reacted less strongly to a simulated predator attack when their paternal grandfather was exposed to predation risk while male F2s were bolder when their maternal grandfather was exposed to predation risk. Therefore, grandpaternal effects were mediated across sexes, from F1 males to F2 females and from F1 females to F2 males. However, these patterns were only evident when one grandfather, but not both grandfathers, were exposed to predation risk. This selective inheritance may mean that grandparental effects are underestimated in the literature and raises new questions about the proximate and ultimate causes of selective transmission across generations.

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

Introduction

Transgenerational plasticity (TGP) occurs when the environment experienced by a parent influences the phenotype of one or more future generations [1]. In some cases, maternal and paternal environments (e.g. predation exposure, diet, stress) have consequences for offspring, but their effects do not persist into future (e.g. F2) generations [2-4]. In other cases, the effects of environments experienced by one generation (F0) persist for multiple generations [5-9], even when offspring (F1s) are raised under 'control' conditions, i.e. in the absence of the cue that triggered a response in the F0 generation. These different patterns of TGP raise questions regarding when and to what degree environmental effects become 'biologically embedded' into the germline and therefore, the extent to which TGP contributes to long-term evolutionary change. Recent evolutionary theory predicts that rates of environmental change influence the likelihood that experiences in one generation have multigenerational consequences [10, 11]. However, this theory largely assumes that inheritance is non-selective (all or nothing). In reality, however, phenotypic changes in the F1 generation may persist selectively across generations in only a subset of individuals via, for example, sex-specific epigenetic changes to chromosomes or gametes [12, 13] that escape erasure at fertilization [14].

Indeed there is some evidence in the biomedical literature that transgenerational effects can persist in a lineage-specific (via either the paternal or maternal lineage) and/or sex-specific (to only male or female F2s) fashion through multiple generations [15-18]. In humans, for instance, grandsons are influenced by the diet of their paternal grandfather while grand-daughters are influenced by the diet of their paternal grandmother [19]. Studies of lineage and sex-specific effects have been conducted almost exclusively in mammals, where mechanisms such as sex-specific placental function and provisioning can generate sex-specific effects [20-22]. It is

unknown if these sex-specific and lineage-specific effects can arise in the absence of such mechanisms (e.g., in external fertilizers). Moreover, our understanding of lineage and sex-specific effects is limited because they are difficult to study, as they require measuring traits in both male and females F2s and tracking effects through both the maternal or paternal lineage (rather than comparing F2s with control grandparents to F2s with two or four experimental grandparents). This is problematic because it leaves us unable to know, for example, whether effects are passed only via either the male or female line (e.g., F0 males to F1 males to F2 males) or whether there are interactive effects across lineages. For example, receiving cues from both the maternal and paternal grandfather may result in different traits or more extreme trait values than receiving cues from only one grandfather. Given that there is strong evidence for interactions between maternal cues, paternal cues, and offspring sex [23-25], it is likely that empirical studies examining interactions between maternal lineage, paternal lineage, and the sex of the F2 generation are important for understanding the evolutionary implications of TGP and the ways in which the environment experienced in the F0 generation manifests in the F2 generation.

Sex-specific and lineage-specific effects may have adaptive significance if they can allow past generations to fine-tune the phenotypes of future generations in response to sex-specific life history strategies or sex differences in the costs and benefits of attending to grandparental cues, which might contain outdated and inaccurate information about the environment. Here, we assessed whether grandpaternal experience with predation risk prior to fertilization affects the traits of their grandoffspring in lineage-specific or sex-specific ways in threespined stickleback (*Gasterosteus aculeatus*). Male and female sticklebacks are sexually dimorphic in several respects, such as habitat use [26] and diet [27]. Further, there are a variety of male-specific

reproductive traits that increase male vulnerability to predation risk [28, 29], including bright nuptial coloration, conspicuous territory defence and courtship behaviour, and paternal care of eggs and newly hatched fry [30]. These sex differences can alter the risks/costs of living in a high predation environment [31], likely altering the optimal phenotype for males versus females in response to cues of predation risk.

In a previous study we exposed male F0s to a cue of predation risk prior to fertilization and found sex-specific paternal effects on offspring brain gene expression and risk-taking behaviour [32]; namely, F1 sons, but not daughters, of predator-exposed fathers were more active relative to sons of control fathers. To understand the extent to which experiences in the F0 generation alter the phenotypes of the F2 generation, in the current study we reared sons and daughters of control and predator-exposed fathers under 'control' conditions and used them to generate F2s with control grandfathers, a predator-exposed maternal grandfather, a predator-exposed paternal grandfather, or two predator-exposed grandfathers (Figure 1). We then assayed male and female F2s for a variety of traits related to predation defence, including behaviour in an open field assay, stress-induced cortisol levels, and body size.

Because mothers and fathers did not interact prior to fertilization nor with their offspring postfertilization, our experimental design allowed us to completely isolate TGP mediated via gametes while controlling for mate choice and differential allocation due to partner quality or parental care. Further, by using artificial fertilization, we controlled for the selective failure of males to court or parent successfully under stressful conditions, which may result in differences between control and predator-exposed lineages because of selective breeding of a nonrandom sample of individuals.

Methods

***Housing conditions*.** In August-September 2016, adult threespined sticklebacks were collected from Putah Creek, a freshwater stream in northern California and shipped to the University of Illinois at Urbana- Champaign. This population has piscivorous predators, including the prickly sculpin (*Cottus asper*). To generate the F1 generation, F0 males were exposed to a clay model sculpin 6 times (over 12 days) or left undisturbed during an equivalent time frame. The day after the last exposure, F1 offspring were generated via *in vitro* fertilization using a split-clutch design: each female's clutch was split and fertilized by both a control and predator-exposed male. Offspring were artificially incubated, reared until adulthood and were not used in any behavioural assays nor exposed to predation risk (see Hellmann, Bukhari [32] for more details).

To generate the F2 generation, we housed adult F1 males singly in 26.5L tanks (36L x 33W x 24H cm), visually isolated from the other males' tanks (August – October 2017). Each tank contained two plastic plants, a sandbox, a clay pot, and algae for nest building. Males were left undisturbed until they had completed their nest, at which point we euthanized the male to obtain sperm. We used a split-clutch design to generate four grandparental treatment groups. Each F1 females' eggs were fertilized by sons of control and predator-exposed fathers; similarly, each F1 male sired eggs from daughters of control and predator-exposed fathers (Figure 1). We successfully generated 32 clutches of half-siblings (some half clutches failed to fertilize or develop): F2s with control grandfathers (n=8 clutches), predator-exposed paternal grandfather (n=8 clutches), predator-exposed maternal grandfather (n=8 clutches), and two predator-exposed grandfathers (n=8 clutches). During this time, the F1 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 bloodworm (*Chironomus* spp.), brine shrimp (Artemia spp.) Mysis shrimp, and cyclopeez.

129 We incubated fertilized eggs in a cup with a mesh bottom placed above an air bubbler
130 and fry were reared in 37.9 L (53L x 33W x 24H cm) tanks, with each half-clutch housed in a
131 separate tank. By artificially fertilizing the eggs and incubating both the F1 and F2 embryos, we
132 controlled for possible pre-fertilization effects mediated by interactions between mothers and
133 fathers as well as the post-fertilization effects mediated by paternal care [33-36]. Offspring were
134 switched to a winter light schedule (8 L: 16 D) at least one month prior to when assays were
135 conducted. Fry were fed newly hatched brine shrimp for two months before transitioning to the
136 mix of frozen food described above.
137
138 ***Open field assays***. When the F2 generation was 5 months (mean days post-hatching:
139 157.9 ± 1.47 s.e.), we measured emergence behaviour, activity, exploration, and antipredator
140 (freezing) behaviour using similar methods described in Hellmann, Bukhari [32]. Briefly, the
141 testing arena was a circular pool (150cm diameter) divided into eight peripheral sections with a
142 circular section in the middle. Fish were placed in an opaque refuge in the centre of the arena
143 with its entrance plugged. After a three minute acclimation period, we removed the plug from the
144 refuge, measured the latency for fish to emerge, and then measured the number of different
145 (exploration) and total (activity) sections visited for three minutes after emergence. Fish that did
146 not emerge after 5 minutes were gently released from the refuge; whether fish emerged naturally
147 or were released did not alter activity/exploration in the resulting periods (generalized linear
148 model with binomial distribution (emerged or released), with activity/exploration difference
149 score (see below) as a fixed effect: Z_{249}=-0.41, p=0.69).
150 After the 3min period, we simulated a predator attack by quickly moving a clay sculpin
151 toward the experimental fish. This attack elicited freezing behaviour from the fish; we measured

the latency for the fish to resume movement and then again measured the number of different and total sections visited for 3 minutes. If the fish remained frozen for greater than 10 minutes (n=25 fish), we ended the trial and considered activity and exploration after the simulated predation attack to be zero. We assayed n=63 F2s with control grandfathers (n=32 females, n=31 males), n=64 F2s with predator-exposed paternal grandfathers (n=35 females, n=29 males), n=61 F2s with predator-exposed maternal grandfathers (n=29 females, n=32 males), n=63 F2s with two predator-exposed grandfathers (n=30 females, n=33 males).

To measure cortisol in response to the predator attack [37], we netted the fish from the arena 15 minutes after the simulated predator attack and quickly weighed and measured it (standard length: from the tip of the nose to the base of the caudal fin). We euthanized the fish in MS-222 and drew blood from the tail of the fish using a heparinized microhematocrit tube. We centrifuged blood to separate the plasma (StatSpin CritSpin Microhemocrit centrifuge) and immediately froze the plasma at -80 °C. Because many fish had non-reproductively mature gonads, we visually sexed offspring when possible; we confirmed the accuracy of this method and sexed the remainder of the fish using a genetic marker [38].

Plasma cortisol. To measure circulating cortisol, we followed the manufacture's protocol (Enzo Life Sciences, Plymouth Meeting, PA, USA). All the plasma samples were prepared in 1:10 steroid displacement reagent solution, then ran with a 1:120 dilution and in duplicate. Slopes of the standard curves and a serial dilution curve (1:20 to 1:320) were parallel (t_6=1.21, p=0.27), indicating that there was negligible matrix interference contributing to systematic measurement error. The intra-assay coefficients of variation were all within acceptable range (3.8%, 2.9%, 4.4%, 4.7%, 4.8%, 3.8%). We ran common samples of pooled plasma on each plate (in

quadruplicate as the first two and last two wells of each plate) to calculate the interassay coefficient of variation (13.9%). Samples with a coefficient of variation greater than 15% (n = 2) were removed from the data set. Due to insufficient amount of blood drawn from some offspring, we sampled n=48 F2s with control grandfathers, n=57 F2s with predator-exposed paternal grandfathers, n=44 F2s with predator-exposed maternal grandfathers, and n=49 F2s with two predator-exposed grandfathers.

Statistical analysis. For the activity and exploration, we found an interaction among observation period (before or after the simulated predator attack), grandmaternal treatment, grandpaternal treatment, and F2 sex. Because of the difficulty interpreting a 4-way interaction, we computed the difference between behaviour before versus after the attack (e.g. sections visited before - visited after the simulated predator attack); see the supplementary material for analysis of the full model with the raw data.

We then used a principal components analysis (R package factoextra) to combine the activity and exploration difference score. We extracted one principle component with an eigenvalue of 1.65 that captured 82.5% of the variation, with smaller values indicating individuals who showed a smaller reduction in activity/exploration after the simulated predator attack compared to before the simulated predator attack. We then used a second principal components analysis to combine latency to emerge from the shelter and latency to resume movement after the simulated predator attack. We extracted one principle component (eigenvalue 1.10) capturing 54.8% of the variation, with high values indicating 'bolder' individuals who were quick to emerge from the shelter and spent little time frozen. We ran activity/exploration and emergence/freezing behaviour as two separate PCAs, rather than one, to

maintain parallelism with our analysis of emergence/freezing behaviour in the F1 generation [32].

To test predictors of variation in activity/exploration, emergence/freezing behaviour, standard length (log-transformed), mass (log-transformed), and stress-induced cortisol (log-transformed), we used MCMC generalized linear mixed models (R package MCMCglmm). Because our data were heteroskedastic, we used 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. All models included fixed effects of maternal grandfather treatment, paternal grandfather treatment, and individual sex. The models testing predictors of activity/exploration, emergence/freezing behaviour, mass, and cortisol also included standard length (log-transformed). The model testing predictors of standard length also included tank density, age (days since hatching), and days since the first clutch hatched, to control for seasonal effects. All models included random effects of mother and father identity nested within maternal and paternal grandfather identity, as well as observer identity for the behavioural data. We tested for possible interactions between maternal grandfather treatment, paternal grandfather treatment, and F2 sex; we retained significant interactions. When significant interactions were present, we investigated those interactions by rerunning the models with male and female F2s analysed separately. We removed three outliers from the mass/length datasets (the same outliers) and two (different) outliers from the cortisol dataset; the significance of the results did not change with these removals.

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

Results

Female F2s were heavier and less responsive to a simulated predator attack when their paternal grandfather, but not both grandfathers, was exposed to predation risk. We sought to understand how grandfathers' (F0) exposure to predation risk influenced the risk-taking behaviour, stress responses, and morphology of individuals in the F2 generation and whether these effects were transmitted in a lineage-specific (via the paternal versus maternal grandfather) and/or sex-specific (to either male versus female F2s) manner (n=251 F2s). We found that both F2 activity/exploration and mass (controlling for length) were influenced by a significant interaction between paternal grandfather treatment, maternal grandfather treatment, and F2 sex (Table 1). Specifically, relative to female F2s with control grandfathers, female F2s with only a predator-exposed paternal grandfather were heavier and showed a reduced change in activity/exploratory behaviour in response to the simulated predator attack; however, these patterns were not present for female F2s with both a paternal and maternal grandfather exposed to predation risk (interaction of maternal by paternal grandfather treatment in female F2s; mass: 95% CI (-0.15, -0.02), p=0.02, Figure 2A; activity/exploration: 95% CI (0.39, 2.14), p=0.005, Figure 2B). For both mass and activity/exploration, we found no evidence of main or interactive effects of maternal or paternal grandfather treatment for male F2s (mass: paternal: 95% CI (-0.12, 0.04), p=0.28, maternal: 95% CI (-0.09, 0.07), p=0.75, interaction: 95% CI (-0.04, 0.13), p=0.27, Figure 2A; activity/exploration: paternal: 95% CI (-0.72, 0.76), p=0.95; maternal: 95%

242 CI (-0.61, 0.90), p=0.70, interaction: 95% CI (-1.41, 0.58), p=0.41, Figure 2B). It is likely that
243 the results for mass and the difference in activity/exploration were similar because they were
244 positively correlated (Wilcoxon signed-rank test: V=22321, p<0.001).
245
246 ***Male F2s were bolder when their maternal grandfather was exposed to predation risk.*** There
247 was a significant interaction between maternal grandfather and paternal grandfather treatment, as
248 well as between maternal grandfather treatment and F2 sex, on boldness (higher values indicate
249 'bolder' individuals who emerged quickly and stayed frozen after the predator attack for shorter
250 periods of time; Table 1). Specifically, male F2s with a maternal grandfather exposed to
251 predation risk were 'bolder' relative to offspring of control grandfathers (95% CI (0.13, 1.20),
252 p=0.02), but this effect tended to be weaker when both the maternal and paternal grandfather
253 were exposed compared to when just the maternal grandfather was exposed (interaction: 95% CI
254 (-1.32, 0.11), p=0.09; Figure 2C). We found no evidence of main or interactive effects of
255 maternal or paternal grandfather treatment for female F2s (paternal: 95% CI (-0.19, 0.94),
256 p=0.18; maternal: 95% CI (-0.48, 0.70), p=0.72; interaction: 95% CI (-1.29, 0.28), p=0.21;
257 Figure 2C).
258
259 ***Neither offspring length, nor stress responses, were significantly altered by grandfathers'***
260 ***predation exposure.*** We found no evidence that stress-induced cortisol varied with paternal
261 (95% CI (-31, 0.29), p=0.95) or maternal (95% CI (-0.56, 0.29), p=0.51) grandfather treatment
262 (n=196 fish). Male F2s had lower stress-induced cortisol than female F2s (95% CI (-0.51, -0.07),
263 p=0.01), but stress-induced cortisol did not vary with length (95% CI (-1.02, 1.92), p=0.56).

We also found no evidence that paternal (95% CI (-0.04, 0.05), p=0.96) or maternal (95% CI (-0.03, 0.06), p=0.41) grandfather treatment altered length of the F2 generation (n=248 fish). Although we found no effect of sex (95% CI (-0.01, 0.03), p=0.46) or age (days since hatched: 95% CI (-0.004, 0.005), p=0.86) on length, F2s were larger when they were in a lower density tank (95% CI (-0.007, -0.003), p<0.001) and when they were born later in the season (95% CI (0.001, 0.004), p=0.002). We found no significant effect of size on activity/exploration or boldness (Table 1).

Discussion

Here, we demonstrate that grandpaternal effects, mediated via sperm, are transmitted selectively to their grandoffspring. Specifically, female F2s were heavier and reacted less strongly to a simulated predator attack when their paternal grandfather was exposed to predation risk. In contrast, male F2s were bolder when their maternal grandfather was exposed to predation risk. For both male and female F2s, this change was only significant when one grandfather, but not both grandfathers, were exposed to predation risk. These findings suggest that grandpaternal effects are both sex-specific and lineage-specific: grandfathers' experiences have different consequences for male and female F2s, and F2 traits depend on whether the paternal grandfather, maternal grandfather, or both grandfathers were exposed to predation risk.

In a previous study, we found striking sex differences in paternal effects in response to predation risk [32]. These sex differences in paternal effects might help explain the lineage-specific effects in the F2 generation that were observed in this study: in Hellmann, Bukhari [32], F1 sons of predator-exposed fathers showed altered activity/exploration and in this study, differences in activity/exploratory behaviour were detected in the descendants of these F1 sons

287 (paternal line). In contrast, activity/exploration of F1 daughters was not affected by their fathers'
288 experience with risk [32] and no changes in activity/exploration were detected in their
289 descendants (maternal line) in this study. More generally, this finding suggests that offspring
290 who do not respond to parental cues may be less likely to transmit information about those cues
291 to future generations.

292 However, we also found that epigenetic transmission and phenotypic consequences can
293 be decoupled: F2 boldness and mass were altered by grandpaternal exposure to predation risk,
294 but not by paternal exposure to predation risk in the F1 generation [32]. Similar results have been
295 found in other systems [39-41], which collectively suggests that individuals may be silent
296 carriers of epigenetic information, transmitting altered phenotypes to their offspring without
297 actually displaying the phenotype themselves. Differences between phenotypes in the F1 and F2
298 generation may be linked to different transmission mechanisms from the F0 to F1 generations
299 compared to the F1 to F2 generations [1, 8]. Alternatively, or in addition, cues from the F0
300 generation may alter how F1s experience their environment (e.g. social interactions [42], habitat
301 choice [43]), which could induce additional epigenetic changes [44] that are transmitted to the F2
302 generation and result in different phenotypes between the F1 and F2 generation. Future work
303 examining differences and similarities in the mechanisms of transmission across multiple
304 generations would be highly useful.

305 Interestingly, we found no evidence for paternal transmission along sex-specific lines
306 (e.g. fathers to sons); rather, we observed the opposite pattern, in which transmission was
307 mediated across sexes from F1 males to F2 females and from F1 females to F2 males. This same
308 pattern of transmission to female descendants (F2s and F3s) via the paternal lineage has been
309 documented in mammals in response to a wide range of maternal experiences, such as high-fat

diets [15], chronic social instability [16], prenatal glucocorticoid exposure [17] and food availability [45]. It is remarkable that we see the same pattern as these mammalian studies, given that the cue originated in a different parent (F0 males versus females), that the mechanism of transmission is almost certainly different (e.g. sperm versus in utero effects), and that the triggering cue varies across studies (e.g. predation risk versus diet). These lineage effects may be generated by a number of different mechanisms including genomic imprinting regulated in a sex-specific manner [15] or sex-specific embryonic responses to differences in sperm content (e.g. small RNAs). An interesting possibility is that epigenetic changes to sex chromosomes are more faithfully transmitted via the F1 heterogametic sex (often males) due to lower rates of sex chromosome recombination [45]. Additional lineage-specific studies across a broader range of taxonomic groups, with diverse potential mechanisms of transmission, may determine the frequency of different patterns of transmission, whether these lineage-specific patterns of transmission are adaptive, and if these patterns are driven by mechanistic constraints on epigenetic erasure in males versus females.

In addition to distinct grandpaternal effects via maternal and paternal lineages, we also found strong interactive effects: grandpaternal effects were evident if one grandfather was exposed to predation risk, but not if both grandfathers experienced predation risk. Interestingly, this mirrors the interactive effects between maternal and paternal cues that were observed in the F1 generation: offspring of predator-exposed fathers showed reduced survival against a sculpin predator, but this pattern was not evident when both the mother and father were exposed to predation risk [32]. This suggests that maternal and paternal effects interact, both when mothers and fathers are directly exposed to the triggering environment (transmitted from predator-exposed F0s to the F1 generation) and when mothers and fathers inherit a cue about the

environment from their parents (transmitted from the offspring of predator-exposed parents to the F2 generation). These interactive effects also mean that if we had not isolated effects emerging in the paternal versus maternal lineage (e.g. compared controls to F2s with two predator-exposed grandfathers), we would have erroneously concluded that effects in the F1 generation did not persist until the F2 generation. Consequently, previous studies that have not examined these lineage effects may have underestimated the extent to which transgenerational effects persist to the F2 generation.

Our experimental design allowed us to control for mate choice and differential allocation during gestation and parental care, which might underlie the sex-specific and lineage-specific effects observed in mammals in response to maternal experiences [20-22]. Here, we demonstrate that similar sex-specific and lineage-specific effects are observed when cues are transmitted via gametes alone (in the absence of these other mechanisms) and when cues are originally experienced by the father instead of the mother. This suggests that these lineage-specific patterns are robust, occurring across a variety of taxonomic groups and mechanisms, and may evolve in response to sex-specific life history strategies. This selective inheritance has significant implications for theory, raising new questions such as how and whether sex-specific selection pressures shape the evolution of transgenerational plasticity, the mechanisms underlying selective transmission of transgenerational information, and whether the mechanism of selective transmission affects the persistence of environmental effects across generations.

Authors contributions

JKH and AMB conceived of the study, JKH collected the data, ERC processed the cortisol samples, JKH analysed the data and wrote the first draft of the manuscript, and JKH and AMB edited the manuscript.

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Table 1: Results of MCMCglmms testing predictors of activity/exploratory behaviour (higher values showed a greater decrease in activity/exploration after the simulated predator attack; n=251 fish), boldness (higher values are individuals that quickly emerged from the shelter and spent little time frozen; n=251 fish), and offspring mass (log-transformed) at 5 months (n=248 fish). We tested fixed effects of maternal and paternal grandfather treatment, F2 sex, and standard length, with random effects of maternal and paternal identity nested within maternal and paternal grandfather, respectively. We also included observer identity in the behavioural models.

	Activity and exploration behaviour		
	Mean	*95% CI (L, U)*	p
Maternal grandfather treatment	-0.37	-1.07, 0.29	0.28
Paternal grandfather treatment	-0.83	-1.48, -0.17	**0.01**
F2 sex	-0.21	-0.84, 0.43	0.51
Standard length (log-transformed)	-0.24	-2.05, 1.57	0.79
Maternal GF * paternal GF	1.09	0.20, 2.02	**0.02**
Paternal GF * F2 sex	0.77	-0.12, 1.69	0.09
Maternal GF * F2 sex	0.40	-0.50, 1.32	0.39
Maternal GF * paternal GF * F2 sex	-1.37	-2.67, -0.10	**0.04**
	Emergence and freezing behaviour		
	Mean	*95% CI (L, U)*	p
Maternal grandfather treatment	0.12	-0.35, 0.59	0.61
Paternal grandfather treatment	0.40	0.001, 0.80	**0.046**
F2 sex	0.16	-0.19, 0.51	0.36
Standard length (log-transformed)	0.66	-0.74, 2.11	0.36
Maternal * paternal GF treatment	-0.55	-1.08, -0.05	**0.035**
Maternal GF * F2 sex	0.51	0.001, 1.01	**0.047**
	Mass (log-transformed)		
	Mean	*95% CI (L, U)*	p
Maternal grandfather treatment	-0.008	-0.07, 0.07	0.99
Paternal grandfather treatment	0.048	-0.02, 0.12	0.18
F2 sex	0.05	0.005, 0.10	**0.03**
Standard length (log-transformed)	2.76	2.60, 2.92	**<0.001**
Maternal GF * paternal GF	-0.07	-0.13, 0.002	0.06
Paternal GF * F2 sex	-0.01	-0.17, -0.03	**0.004**
Maternal GF * F2 sex	-0.17	-0.08, 0.05	0.62
Maternal GF * paternal GF * F2 sex	0.11	0.02, 0.21	**0.02**

Figure Legend

Figure 1: Males in the F0 generation were either left unexposed (white) or directly exposed to predation risk (dark grey) and their sperm was used to fertilize the eggs of an unexposed female using in vitro fertilization. The F1 generation was reared in the absence of predation risk and used to generate the F2 generation. For example, sons of predator-exposed fathers were mated to daughters of control fathers to generate F2s with a predator-exposed paternal grandfather. Similarly, daughters of predator-exposed fathers were mated to sons of control fathers to generate F2s with a predator-exposed maternal grandfather. Light grey indicates F1s/F2s whose lineage was exposed to predation risk (i.e. their parents or grandparents experienced predation risk). Juvenile F2s were then assayed for a variety of traits.

Figure 2: A) Mass (log-transformed) of female (red, circles) and male (blue, triangles) F2s with control grandfathers, predator-exposed paternal grandfather, predator-exposed maternal grandfather, or two predator-exposed grandfathers (5 months post-hatching). Plotted on the y-axis are the residuals of the regression model, with grandparental treatment and sex removed (mean ± s.e.). B) Difference score in activity/exploratory behaviour (PCA) of female and male F2s, with higher values indicate individuals who showed a greater change in activity/exploration behaviour before versus after the predator attack (mean ± s.e.) in the open field assay. All treatment groups were less active/exploratory after the simulated predator attack compared to before (Supplementary Figure 1). C) Emergence time and freezing behaviour (PCA) of female and male F2s across the four grandpaternal treatments. Higher values indicate 'bolder' individuals who were quick to initially emerge from the shelter and who spent a reduced amount of time frozen after the simulated predator attack (mean ± s.e.).

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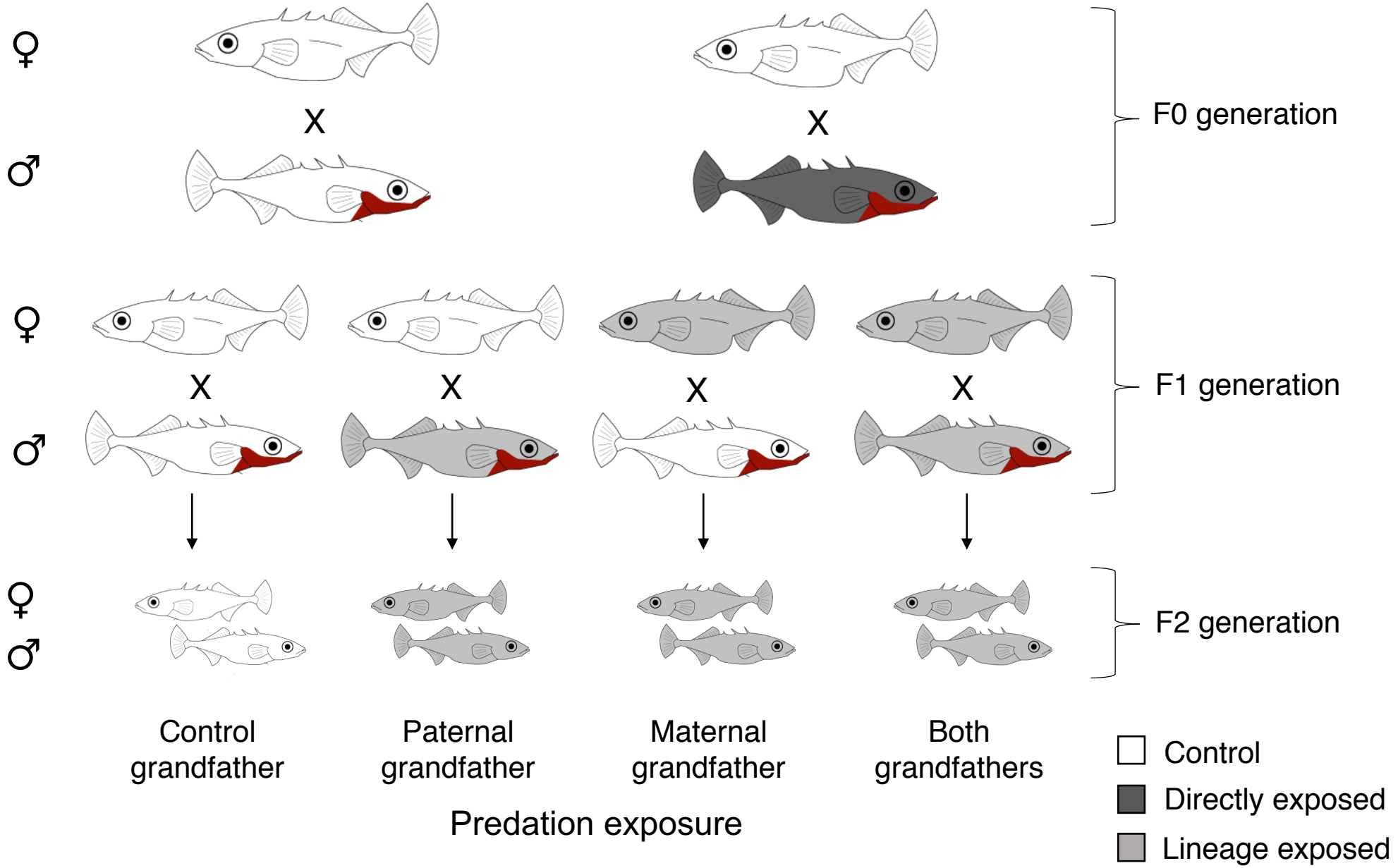
♀
♂
X
X
F0 generation
♀
♂
X
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X
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F1 generation
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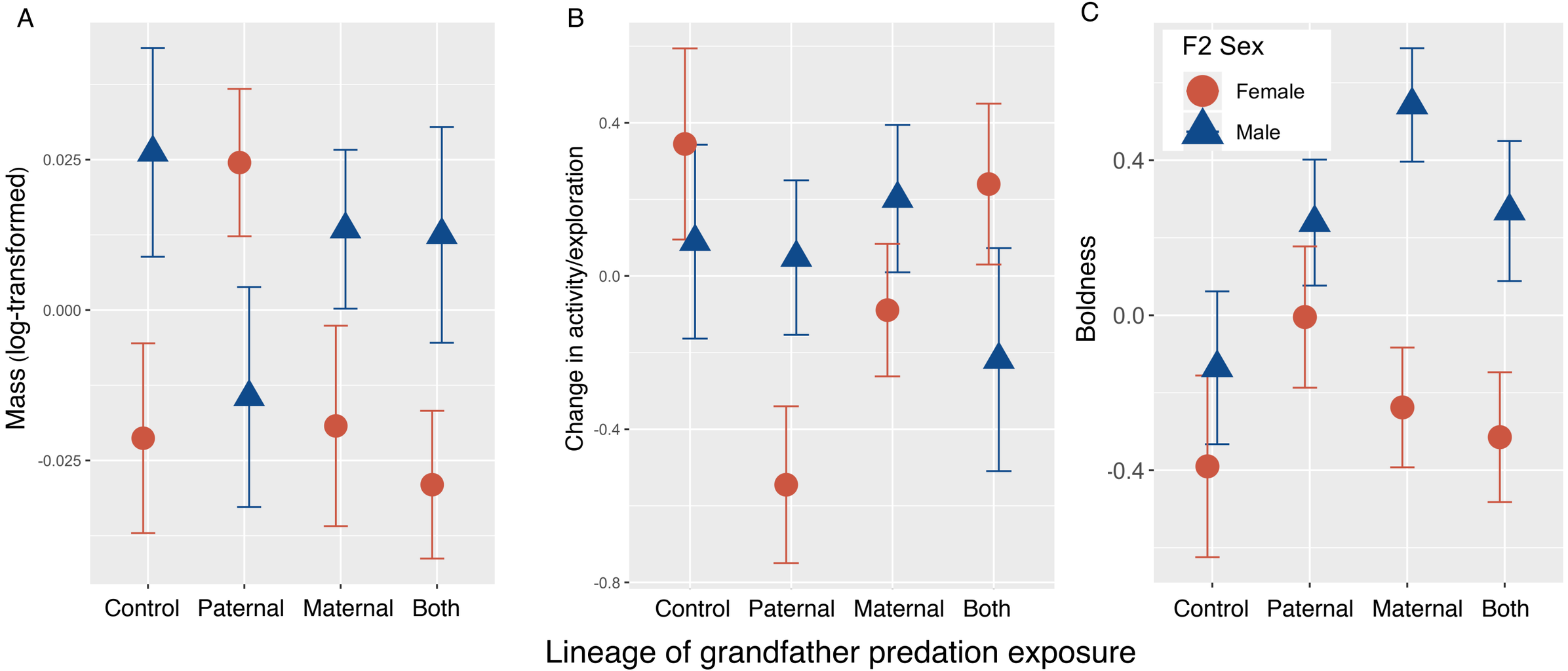
F2 generation
Control grandfather
Paternal grandfather
Maternal grandfather
Both grandfathers
Predation exposure
Control
Directly exposed
Lineage exposed


A
Mass (log-transformed)
0.025
0.000
-0.025
Control
Paternal
Maternal
Both
B
Change in activity/exploration
0.4
0.0
-0.4
-0.8
Control
Paternal
Maternal
Both
C
Boldness
0.4
0.0
-0.4
Control
Paternal
Maternal
Both
F2 Sex
Female
Male
Lineage of grandfather predation exposure