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

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Abstract

1. 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). Sex selective TGP, via specific lineages or to only male or female descendants, has been underexplored in natural systems, but may be adaptive if it allows past generations to fine-tune the phenotypes of future generations in response to sex-specific life history strategies.
2. We sought to understand if exposing males to predation risk can influence grandoffspring via sperm in threespined stickleback (*Gasterosteus aculeatus*). We specifically tested the hypothesis that grandparental effects are transmitted in a sex-specific way down the male lineage, from paternal grandfathers to F2 males.
3. We reared F1 offspring of unexposed and predator-exposed F0 males 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. We then assayed male and female F2s for a variety of traits related to antipredator defense.
4. Grandpaternal effects depended on lineage and were mediated largely across sexes, from F1 males to F2 females and from F1 females to F2 males. When their paternal grandfather was exposed to predation risk, female F2s were heavier and showed a reduced change in behavior in response to a simulated predator attack relative to offspring of unexposed grandparents. In contrast, male F2s showed reduced antipredator behavior when their maternal grandfather was exposed to predation risk. However, these patterns were only evident when one grandfather, but not both grandfathers, was exposed to predation risk.
5. If sex-specific effects are common, then grandparental effects are likely underestimated in the literature. These results draw attention to the importance of sex-selective inheritance of environmental effects and raise new questions about the proximate and ultimate causes of selective transmission across generations.

Key words: phenotypic plasticity, paternal effect, *Gasterosteus aculeatus*, nongenetic inheritance, intergenerational plasticity, predation risk, transgenerational plasticity

Introduction

Transgenerational plasticity (TGP, environmental parental effects) occurs when the environment experienced by a parent influences the phenotype of one or more future generations (Bell & Hellmann 2019). Although the importance of maternal environments for offspring is well-appreciated (Bonduriansky & Day 2008; Sheriff *et al.* 2017), recent studies are showing that paternal environments such as stress (Rodgers *et al.* 2013; Dias & Ressler 2014; Gapp *et al.* 2014), diet (Ng *et al.* 2010; de Castro Barbosa *et al.* 2016), immune priming (Beemelmanns & Roth 2016) and thermal changes (Weyrich *et al.* 2016; Sales *et al.* 2018) also have consequences for offspring, and fathers can influence their offspring via epigenetic changes to sperm (e.g., small RNAs (Rodgers *et al.* 2015; Immler 2018)). In some cases, parental environments influence the phenotypes of the F1 generation but their effects do not persist into future (e.g. F2) generations (Remy 2010; Wibowo *et al.* 2016; Zhou *et al.* 2018). However, there is also evidence that the effects of environments experienced by one generation (F0) can persist for multiple generations (Kaati, Bygren & Edvinsson 2002; Dias & Ressler 2014; Gapp *et al.* 2014; Cropley *et al.* 2016; Beemelmanns & Roth 2017), 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 (grandparental effects). Grandparental effects can be adaptive when 1) rates of environmental change are low such that grandparental environments are good predictors of the environments that grand-offspring will encounter (Dall, McNamara & Leimar 2015; Leimar & McNamara 2015), 2) there is a low cost of false positives (e.g., maintaining a predator-induced phenotype in a benign environment) such that there is weak selection against maintaining the induced phenotype and 3) there is a high cost of false negatives (e.g., having a 'safe' phenotype in a risky

environment (Sheriff *et al.* 2018)), such that there is strong selection against losing the induced phenotype.

Current theory on TGP largely assumes that multigenerational inheritance is non-selective, i.e. that all grandoffspring are equally affected by their grandparents' environments. However, in reality, phenotypic changes in the F1 generation may persist selectively across generations in only a subset of individuals in, for example, a sex-specific manner. Indeed there is some evidence 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 (Dunn & Bale 2011; Saavedra-Rodríguez & Feig 2013; Bygren 2014; Moisiadis *et al.* 2017). 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 (Pembrey *et al.* 2006). Multigenerational studies examining both lineage and sex-specific effects have been conducted almost exclusively in mammals (but see (Emborski & Mikheyev 2019)), where mechanisms such as sex-specific placental function and provisioning can generate sex-specific effects in the F1 generation (Bale 2011; Glover & Hill 2012; Yun, Lee & Kim 2016) that might affect the ways in which these effects are transmitted to the F2 generation. It is unknown if sex-specific and lineage-specific effects can arise in externally-fertilizing organisms, or organisms that lack parental care, via mechanisms such as sex-specific epigenetic changes to chromosomes or gametes (Lemos *et al.* 2013; Francisco & Lemos 2014) that escape erasure at fertilization (Heard & Martienssen 2014).

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.

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. However, recent theoretical work has shown that nonadaptive sex-specific parental effects may arise due to sexual conflict, especially in systems with strong sexual selection (Burke, Nakagawa & Bonduriansky 2019).

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 (Reimchen 1980) and diet (Reimchen & Nosil 2001). Further, there are a variety of male-specific reproductive traits that increase male vulnerability to predation risk (Candolin 1998; Johnson & Candolin 2017), including bright nuptial coloration, conspicuous territory defense and courtship behavior, and paternal care of eggs and newly hatched fry (Bell & Foster 1994). These sex differences can alter the risks/costs of living in a high predation environment (Reimchen & Nosil 2004), likely altering the optimal phenotype for males versus females in response to cues of predation risk. Because fathers and

their male descendants share the same sex-specific selective pressures, we specifically sought to test the prediction that these sex-specific differences in life history strategies may select for inheritance down male lineages: specifically, that experiences of F0 males would be transmitted to F1 males to F2 males, but not to F2 females (significant paternal lineage by F2 sex interaction).

To understand the extent to which cues of predation risk in the F0 generation altered the phenotypes of the F2 generation, we reared sons and daughters of control and predator-exposed fathers under 'control' conditions (Hellmann *et al.* in review) 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). For paternal effects, only phenotypic changes in the F2 generation and beyond are considered truly transgenerational, as any environment experienced by F0 males simultaneously exposes both the father (F0) and his germline (future F1) (Heard & Martienssen 2014). We then assayed male and female F2s to determine if grandpaternal predation exposure induced traits related to antipredator defense (Bell, McGhee & Stein 2016; Sheriff *et al.* 2017), including increased antipredator behaviors in an open field assay as well as altered stress-induced cortisol levels and body size. This is, to our knowledge, the first manipulative study to examine both sex and lineage-specific grandpaternal effects (i.e. through both the maternal versus paternal grandfather), and will allow us to understand whether sperm-mediated paternal effects can be transmitted across generations in patterns that are similar to those observed with respect to grandmaternal effects.

Methods

***Housing conditions*.** In June-September 2016, adult, sexually mature 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*). The F0 generation was maintained on a summer photoperiod schedule (16 L : 8D) at 21° ± 1°C and fed ad libitum daily with a mix of frozen bloodworms (*Chironomus* spp.), brine shrimp (Artemia spp.) Mysis shrimp, and Cyclop-eeze.

To simulate breeding conditions, where males defend nesting territories while females shoal together, F0 females were housed in groups of n=10 fish per tank while F0 males were isolated in a 26.5L tanks (36L x 33W x 24H cm) with nesting materials; once F0 males had successfully built a nest, they were exposed to a clay model sculpin (21cm long) 6 times over 11 days or left undisturbed during an equivalent time frame (n=16 F0 males total). This predator exposure regimen was designed to mimic conditions that males experience when they move into shallow habitats to nest. We elected to expose males to a relatively short stressor in order to minimize the potential for males to habituate to the model predator (Dellinger *et al.* 2018) and to minimize the possibility of influencing sperm quality; although stickleback males produce sperm in the beginning of the breeding season (Borg 1982), it is possible that a prolonged stressor such as simulated predation risk could influence sperm production and/or quality.

The day after the last exposure, F1 offspring were generated via *in vitro* fertilization using a split-clutch design: an unexposed, wild-caught female's clutch was split and fertilized by both a control and predator-exposed male. We incubated fertilized F1 eggs in a cup with a mesh bottom placed above an air bubbler and fry were reared until adulthood in 37.9 L (53L x 33W x 24H cm) tanks, with each half-clutch housed in a separate tank (densities ranging from 4-28 individuals per tank). The F1 generation was fed newly hatched brine shrimp for 2 months before transitioning to the same mix of frozen bloodworms, brine shrimp, Mysis shrimp, and Cyclop-

eeze noted above. The F1 generation was switched from a summer photoperiod schedule (16 L : 8D) at 21° ± 1°C to a winter light schedule (8 L: 16 D) at 20° ± 1°C at the end of the breeding season (December 2016). The F1s used in this experiment were not used in any behavioral assays nor exposed to predation risk (see Hellmann *et al.* (in review) for more details- F1s used in this experiment were siblings of the F1s with control mothers used in Hellmann *et al.* (in review)).

Once the F1 generation neared reproductive maturity (May 2017), the F1 generation was switched from a winter photoperiod (8 L: 16 D at 20° ± 1°C) to a summer photoperiod schedule (16 L : 8D) at 21° ± 1°C. From August – October 2017, we bred male and females F1s to generate the F2 generation. We isolated adult F1 males in 26.5L tanks (36L x 33W x 24H cm); 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. F1 females were maintained in their home tanks (to mimic shoaling in the wild) and fed twice per day to encourage egg production; once females were gravid, we gently squeezed their abdomen to obtain eggs. 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). To avoid inbreeding, mated F1s had different F0 mothers and fathers. We successfully generated 32 clutches of half-siblings: 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). In addition to the 32 successful clutches, we had n=10 half clutches that failed to fertilize/hatch (n=4 control, n=2 paternal grandfather, n=2 maternal grandfather, n=2 both

grandfathers). In total, the F2 generation used in the assays below were descendants of n = 16 F0 grandfathers and n = 19 F1 fathers (range of n = 7-11 F2s tested per clutch, plus one clutch where only n =2 F2s were tested).

As with the F1 generation, we incubated fertilized eggs with an air bubbler and fry were reared in 37.9 L (53L x 33W x 24H cm) tanks, with each half-clutch housed in a separate tank (mean 18.7 individuals per tank, range: 6-35). Offspring were switched to a winter light schedule (8 L: 16 D) at least one month prior to when assays were conducted. As with the F1 generation, F2 fry were fed newly hatched brine shrimp for two months before transitioning to the mix of frozen food described above.

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 (Mashoodh *et al.* 2012; Stein & Bell 2014; McGhee *et al.* 2015; Mashoodh *et al.* 2018). 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.

Open field assays. When the F2 generation was 5 months old (mean days post-hatching: 157.9, range 146-169 days), we measured activity/exploration, and antipredator behavior (freezing and evasive swimming) using similar methods described in Hellmann *et al.* (in review). Briefly, the testing arena was a circular pool (150cm diameter) divided into eight peripheral sections with a circular section in the middle (using lines drawn on the base of the pool). Fish were placed in an

opaque refuge in the center of the arena with its entrance plugged. After a three minute acclimation period, we removed the plug from the refuge, allowed 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 5 minutes after the plug was removed 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_{249}=-2.39, p=0.01), controlling for emergence time did not alter the significance of the activity/exploration results reported below.

After the 3min period, we simulated a predator attack by quickly moving a clay sculpin toward the experimental fish for approximately 5 seconds and then removing the sculpin from the arena. This attack often elicited evasive (jerky) swimming behavior followed by freezing behavior from the fish (we interpret increased time frozen and evasive swimming as greater antipredator behavior); we measured whether or not fish performed the evasive swimming behavior as well as the latency for the fish to resume movement. After the fish resumed movement, we 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), and n=63 F2s with two predator-exposed grandfathers (n=30 females, n=33 males). Assays were scored live, by an observer who was standing at least 1m from the pool.

To measure cortisol in response to the predator attack (Mommer & Bell 2013), 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 dissected fish and identified sex based on the presence of immature testes and ovaries; we confirmed the accuracy of this method and sexed any questionable fish using a genetic marker (Peichel *et al.* 2004).

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 used a principal components analysis (R package factoextra (Kassambara & Mundt 2017)) to combine the activity and exploration (Spearman rank correlation of activity and exploration: $\rho=0.91$, $p<0.001$**)**. 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 one principal component with an eigenvalue of 1.75 that captured 87.5% of the variation, with larger values indicating individuals who were more active/explorative.

To test predictors of activity/exploration, antipredator behaviors (evasive swimming, freezing), standard length (log-transformed), mass (log-transformed), and stress-induced cortisol (log-transformed), we used MCMC generalized linear mixed models (R package MCMCglmm (Hadfield 2010)). We ran separate models for male and female F2s because of our a priori hypotheses regarding male-lineage effects (i.e. that F2 male phenotypes would be altered by the experiences of their paternal grandfather) and to avoid higher order interaction terms (maternal grandfather by paternal grandfather by F2 sex interactions) that are difficult to interpret.

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, and thin = 3. We used a Gaussian distribution for activity/exploration, standard length, mass, and cortisol, a Poisson distribution for freezing behaviour, and a categorical distribution for evasive swimming behavior. All models included fixed effects of maternal and paternal grandfather treatment. The models testing predictors of activity/exploration, emergence/freezing behavior, mass, and cortisol also included standard length (log-transformed). The model testing predictors of standard length included tank density (assessed at 4.5 months) and age (days since hatching). Because

there were two observations per individual, the model of activity-exploration included random effects of fish ID nested within maternal and paternal identity nested within maternal and paternal grandfather (respectively). The remaining models all included random effects of mother and father identity nested within maternal and paternal grandfather identity, as well as observer identity for the behavioral data. We tested for possible interactions between maternal and paternal grandfather treatment (as well as interactions with observation period for the activity/exploration model); we retained significant interactions. When significant interactions were present, we investigated those interactions by rerunning the models with grandfather treatment as a 4-factor variable to determine differences between F2s of control grandparents and F2s with either maternal, paternal, or two experimental grandfathers.

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 less responsive to a simulated predator attack when their paternal grandfather, but not both grandfathers, was exposed to predation risk. We sought to test the hypothesis that grandparental effects are transmitted in a sex-specific way down the male lineage, from paternal grandfathers to F2 males. For activity/exploration, we found no evidence of main or interactive effects of maternal or paternal grandfather treatment for male F2s (Table 1; Figure 1). For female F2s, we found that activity/exploration was influenced by a significant three-way interaction between paternal grandfather treatment, maternal grandfather treatment,

and observation period (Table 1). Specifically, female F2s with a predator-exposed paternal grandfather showed a reduced change in activity/exploratory behavior in response to the simulated predator attack compared to female F2s with control grandfathers (significant paternal GF by observation period interaction: 95% CI (-0.34, 1.66), p=0.003; Figure 1); however, this interaction was not significantly different than the controls for female F2s with either a predator-exposed maternal grandfather (95% CI (-0.20, 1.18), p=0.17) or two predator-exposed grandfathers ((95% CI (-0.56, 0.81), p=0.75; Figure 1).

Male F2s showed reduced antipredator behaviors when one grandfather, but not both grandfathers, was exposed to predation risk. For male F2s, there was a significant interaction between maternal grandfather and paternal grandfather treatment on both freezing behavior and evasive swimming behaviors (Table 1). Specifically, male F2s with a maternal grandfather exposed to predation risk were less likely to perform evasive swimming behaviors (95% CI (-446, 5), p=0.04) after the simulated predator attack relative to offspring of control grandfathers. However, there was no significant difference between male F2s with a control grandfather and male F2s with either a predator-exposed paternal grandfather (95% CI (-233, 164), p=0.75) or two predator-exposed grandfathers (95% CI (-138, 268), p=0.51). Similarly, male F2s with a paternal grandfather exposed to predation risk tended to spend less time frozen (95% CI (-1.85, 0.10), p=0.08) after the simulated predator attack relative to offspring of control grandfathers, but this was not true for male F2s with a control grandfather and male F2s with either a predator-exposed maternal grandfather (95% CI (-1.74, 0.33), p=0.19) or two predator-exposed grandfathers (95% CI (-4.74, 1.58), p=0.30). We found no evidence of main or interactive effects of maternal or paternal grandfather treatment for female F2 evasive swimming or freezing

behaviors (Table 1; Figure 2C). There was no correlation between the length of time frozen and whether or not individuals performed evasive swimming behaviors (t_{247}=-0.58, p=0.56).

Female F2s were heavier when their paternal grandfather, but not both grandfathers, was exposed to predation risk. For female F2s, we found a significant interaction between maternal grandfather and paternal grandfather treatment on mass (Table 2). Specifically, female F2s tended to be heavier when either their paternal **(**95% CI (-0.01, 0.21), p=0.07) or maternal **(**95% CI (-0.01, 0.21), p=0.07) grandfather was exposed to predation risk, but not when both grandfathers were exposed to predation risk **(**95% CI (-0.11, 0.15), p=0.79). Male F2 mass was not altered by maternal or paternal grandfather treatment (Table 2; Figure 3). We found no evidence that stress-induced cortisol or length varied with paternal or maternal grandfather treatment for either male or female F2s (Table 2). Although we found no effect of age on length, F2s were larger when they were in a lower density tank (Table 2). We found no significant effect of size on activity/exploration, freezing behavior, or stress-induced cortisol (Table 1, 2).

Discussion

Here, we demonstrate that grandpaternal effects, mediated via sperm, are transmitted selectively to their grandoffspring. Specifically, female F2s reacted less strongly to a simulated predator attack when their paternal grandfather was exposed to predation risk and tended to be heavier when either their maternal or paternal grandfather was exposed to predation risk. In contrast, male F2s were less likely to perform evasive swimming (antipredator) behavior when their maternal grandfather was exposed to predation risk and tended to spend less time frozen when their paternal grandfather was exposed to predation risk. For all the above patterns, this

change was present 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.

Interestingly, we found weak evidence for our hypotheses that paternal transmission would occur along sex-specific lines (e.g. fathers to sons); rather, we largely observed the opposite pattern, in which transmission was mediated across sexes from F1 males to F2 females and from F1 females to F2 males. Emborski and Mikheyev (2019) also found multigenerational transmission across sexes in fruit flies, whereby male offspring were influenced by ancestral maternal diet where female offspring were influenced by ancestral paternal diet. Further, a similar pattern of transmission to female descendants (F2s and F3s) via the paternal lineage has been documented in mammals in response to a wide range of maternal experiences, such as high-fat diets (Dunn & Bale 2011), chronic social instability (Saavedra-Rodríguez & Feig 2013), prenatal glucocorticoid exposure (Moisiadis *et al.* 2017) and food availability (Bygren *et al.* 2014). It is interesting that we see a similar pattern of transmission from the F1 to the F2 generations as these mammalian studies, given that the cue originated in a different parent (F0 males versus females) and that the triggering cue varies across studies (e.g. predation risk versus diet).

These sex-specific patterns may arise because of mechanistic constraints: the underlying proximate mechanism may set limits on how epigenetic information can be transmitted across generations, given widespread epigenetic erasure that occurs at fertilization in most vertebrates (Heard & Martienssen 2014). These lineage effects may be generated by a number of different

mechanisms including genomic imprinting regulated in a sex-specific manner (Dunn & Bale 2011) or sex-specific embryonic responses to differences in sperm content (e.g. small RNAs). It is possible that some patterns of transmission might be more faithful than others. For example, 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 (Bygren *et al.* 2014).

Sex-specific and lineage specific effects may also reflect life history differences between males and females, which might affect both 1) how the F1 generation perceive their environment and transmit cues of predation risk and 2) the different costs and benefits of attending to potentially outdated information from grandparents. For example, in species with high reproductive variance among males (such as sticklebacks), males may face a high cost of false positives (e.g., maintaining a predator-induced phenotype in a benign environment), as mismatches between their phenotype and their environment may reduce their ability to compete with higher quality males. However, the direction of these effects- that F2s showed reduced antipredator behaviors and a reduced change in behavior in response to a simulated predator attack- suggest that F2s with predator-exposed grandfathers are actually less responsive to predation risk. This is consistent with the findings of Sentis *et al.* (2018) which found that, although aphid mothers exposed to predation risk produced many winged F1 morphs to facilitate dispersal, the frequency of winged morphs in the F2 generation was lower than the control group. It is currently unclear if this low antipredator defense in the F2 generation is adaptive (potentially driven by negative maternal effects (Kuijper & Hoyle 2015)) or non-adaptive.

Indeed, it is possible that these sex-specific and lineage effects are not adaptive. Burke, Nakagawa and Bonduriansky (2019) suggest that complex sex-specific patterns may be

particularly likely to be non-adaptive, because they reflect intralocus conflict that results from sex-specific selective pressures. These non-adaptive effects may be particularly likely to arise in species in which males are subject to strong sexual selection, as is the case in sticklebacks where there is high competition for breeding sites and females. Consequently, 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 and whether these lineage-specific patterns of transmission are adaptive and/or if driven by mechanistic constraints.

In addition to distinct grandpaternal effects via maternal and paternal lineages, we also found interactive effects: grandpaternal effects were evident if one grandfather was exposed to predation risk, but not if both grandfathers experienced predation risk. 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. This suggests that previous studies that have not examined these lineage effects may have underestimated the extent to which transgenerational environmental effects persist to the F2 generation. Interestingly, these lineage effects mirror 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 (Hellmann *et al.* in review). 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 patterns may result because the combination of two parents/grandparents exposed to predation risk produces an entirely different phenotype compared to one parent/grandparent (Lehto & Tinghitella 2020; Hellmann *et al.* in review), resulting in different phenotypic changes in F2s with a maternal, paternal, or both grandfathers exposed to predation risk.

In our results, we also found that epigenetic transmission and phenotypic consequences can be decoupled: F2 antipredator behavior and mass were altered by grandpaternal exposure to predation risk, but not by paternal exposure to predation risk in the F1 generation (Hellmann *et al.* in review). Similar results have been found in other systems (Panacek *et al.* 2011; Kim, Kwak & An 2013; Crocker & Hunter 2018), which collectively suggests that individuals may be silent carriers of epigenetic information, transmitting altered phenotypes to their offspring without actually displaying the phenotype themselves. Differences between phenotypes in the F1 and F2 generation may be linked to different proximate mechanisms that result in altered phenotypes in the F1 generations compared to F2 generations (Gapp *et al.* 2014; Bell & Hellmann 2019). For example, the F0 germline (sperm that will sire the F1 generation) is directly exposed to predation risk whereas the F1 germline is unexposed, potentially leading to different epigenetic changes in the F0 versus F1 sperm (Gapp *et al.* 2014). Alternatively, or in addition, cues from the F0 generation may alter how F1s experience their environment (e.g. social interactions (Giesing *et al.* 2011), habitat choice (Bestion *et al.* 2014)), which could induce additional epigenetic changes (Nagy & Turecki 2012) that are transmitted to the F2 generation and result in different phenotypes between the F1 and F2 generation. Future work examining differences and similarities in the mechanisms of transmission across multiple generations would be highly useful.

This is the most comprehensive study to date on sex and lineage specific paternal effects and shows that these patterns are complex and potentially non-adaptive. The majority of the literature on sex-specific and lineage-specific transgenerational plasticity has been carried out on maternal effects in mammals. In those systems, it is possible that mate choice and differential allocation during gestation and parental care could cause the observed sex-specific and lineage-specific effects. Here, we demonstrate that similar sex-specific and lineage-specific effects can occur in different taxonomic groups (fish versus mammals), are observed when cues are transmitted via gametes alone (in the absence of *in utero* effects, parental care or differential allocation), and when cues are originally experienced by the father instead of the mother. 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.

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Authors contributions

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

Data Accessibility

All datasets (behavior, morphology, cortisol) will be made available on Dryad upon acceptance of this manuscript.

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Table 1: Results of MCMC GLMMs testing predictors of F2 activity/exploratory behavior (higher values showed more active and exploratory individuals; n=125 males, n=126 females), freezing behavior (n=125 males, n=126 females), and evasive swimming behavior (n=124 males, n=125 females). We tested fixed effects of maternal and paternal grandfather (GF) treatment, and standard length, with observation period (before or after the simulated predator attack) in the activity/exploration model only. All models included random effects of observer identity; the models of freezing behavior and evasive swimming included maternal and paternal identity nested within maternal and paternal grandfather (respectively) and the model of activity/exploration included random effects of fish ID nested within maternal and paternal identity nested within maternal and paternal grandfather (respectively).

	Female F2s			**Male F2s**		
	Activity and exploration behavior					
	Mean	*95% CI (L, U)*	p	*Mean*	*95% CI (L, U)*	p
Maternal grandfather treatment	-0.49	-1.36, 0.37	0.25	-0.01	-0.63, 0.64	0.96
Paternal grandfather treatment	-0.62	-1.34, 0.06	0.08	-0.10	-0.54, 0.34	0.64
Observation period	-1.33	-1.80, -0.85	**<0.001**	-0.98	-1.24, -0.72	**<0.001**
Standard length (log-transformed)	0.57	-1.57, 2.72	0.60	1.11	-0.55, 2.78	0.19
Maternal GF * paternal GF	1.09	0.21, 1.97	**0.02**	---	---	---
Paternal GF * observation period	1.01	0.34, 1.67	**0.003**	---	---	---
Maternal GF * observation period	0.49	-0.22, 1.16	0.17	---	---	---
Maternal GF * paternal GF * obs period	-1.38	-2.35, -0.43	**0.005**	---	---	---
	Freezing behavior					
	Mean	*95% CI (L, U)*	p	*Mean*	*95% CI (L, U)*	p
Maternal grandfather treatment	-0.24	-0.90, 0.43	0.46	-0.72	1.78, 0.32	0.16
Paternal grandfather treatment	0.04	-0.62, 0.70	0.89	-0.87	-1,77, -0.02	**0.048**
Standard length (log-transformed)	-2.01	-5.36, 1.36	0.24	-1.62	-3.87, 0.55	0.15
Maternal * paternal GF treatment	---	---	---	0.89	0, 1.750	**0.046**
	Evasive swimming behavior					
	Mean	*95% CI (L, U)*	p	*Mean*	*95% CI (L, U)*	p
Maternal grandfather treatment	-120	-329, 63	0.18	-212	-446, 7	**0.04**
Paternal grandfather treatment	-63	-256, 126	0.47	-30	-229, 171	0.75
Standard length (log-transformed)	-689	-356, 1865	0.18	-143	-883, 592	0.69
Maternal * paternal GF treatment	---	---	---	305	17, 607	**0.02**

Table 2: Results of MCMC GLMMs testing predictors of log-transformed F2 mass (n=125 males, n=126 females), log-transformed standard length (n=125 males, n=126 females), and cortisol (n=97 males, n=99 females). For both mass and cortisol, we tested fixed effects of maternal and paternal grandfather (GF) treatment, and standard length (log-transformed). For standard length, we tested fixed effects of maternal and paternal grandfather (GF) treatment, the density of the home tank, and age (the number of days since hatching). All models included random effects of maternal and paternal identity nested within maternal and paternal grandfather (respectively).

	Female F2s			**Male F2s**		
	Mass					
	Mean	*95% CI (L, U)*	p	*Mean*	*95% CI (L, U)*	p
Maternal grandfather treatment	-0.10	-0.01, 0.21	0.07	0.02	-0.07, 0.10	0.69
Paternal grandfather treatment	0.10	-0.01, 0.21	0.07	-0.05	-0.13, 0.03	0.18
Standard length (log-transformed)	1.89	1.54, 2.23	**<0.001**	2.45	2.17, 2.72	**<0.001**
Maternal * paternal GF treatment	-0.18	-0.29, -0.08	**<0.001**	---	---	---
	Standard length					
	Mean	*95% CI (L, U)*	p	*Mean*	*95% CI (L, U)*	p
Maternal grandfather treatment	-0.03	-0.04, 0.09	0.41	0.02	-0.04, 0.09	0.46
Paternal grandfather treatment	0.01	-0.05, 0.08	0.67	0.02	-0.05, 0.09	0.57
Tank density	-0.004	-0.007, -0.014	**0.004**	-0.005	-0.01, -0.001	**0.01**
Days since hatching	0	-0.007, 0.007	0.99	-0.004	-0.01, 0.003	0.27
	Stress-induced cortisol					
	Mean	*95% CI (L, U)*	p	*Mean*	*95% CI (L, U)*	p
Maternal grandfather treatment	-0.22	-0.70, 0.26	0.33	-0.02	-0.56, 0.51	0.95
Paternal grandfather treatment	-0.06	-0.41, 0.28	0.72	-0.03	-0.53, 0.45	0.89
Standard length (log-transformed)	-0.54	-2.14, 1.10	0.51	1.72	-0.72, 4.09	0.16

Figures

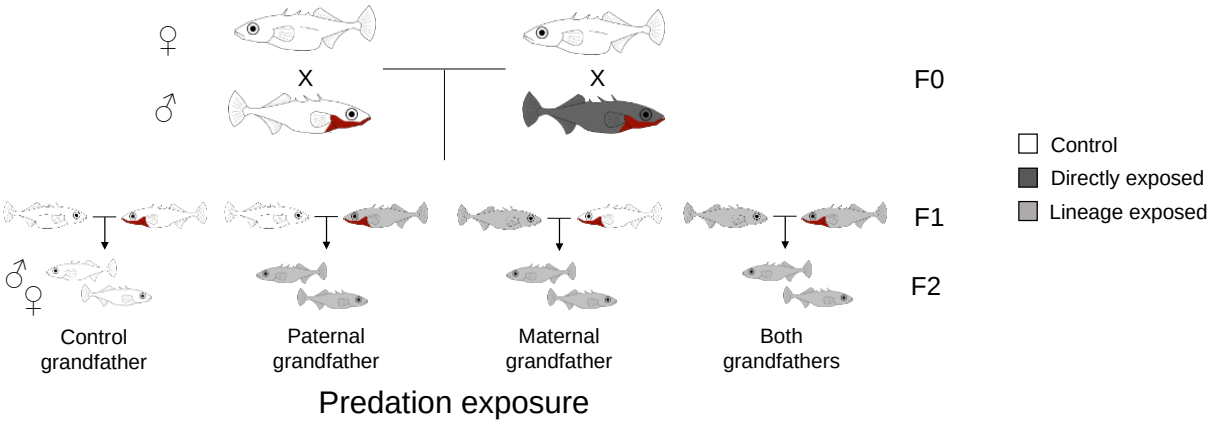

Figure 1: Wild-caught males in the F0 generation were either left unexposed (white) or directly exposed to predation risk (dark grey) and their sperm was used to fertilize the eggs of an unexposed, wild-caught female using in vitro fertilization. The F1 generation was reared in the absence of predation risk and used to generate the F2 generation. For example, F1 sons of predator-exposed fathers were mated to F1 daughters of control fathers to generate F2s with a predator-exposed paternal grandfather. Similarly, F1 daughters of predator-exposed fathers were mated to F1 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.

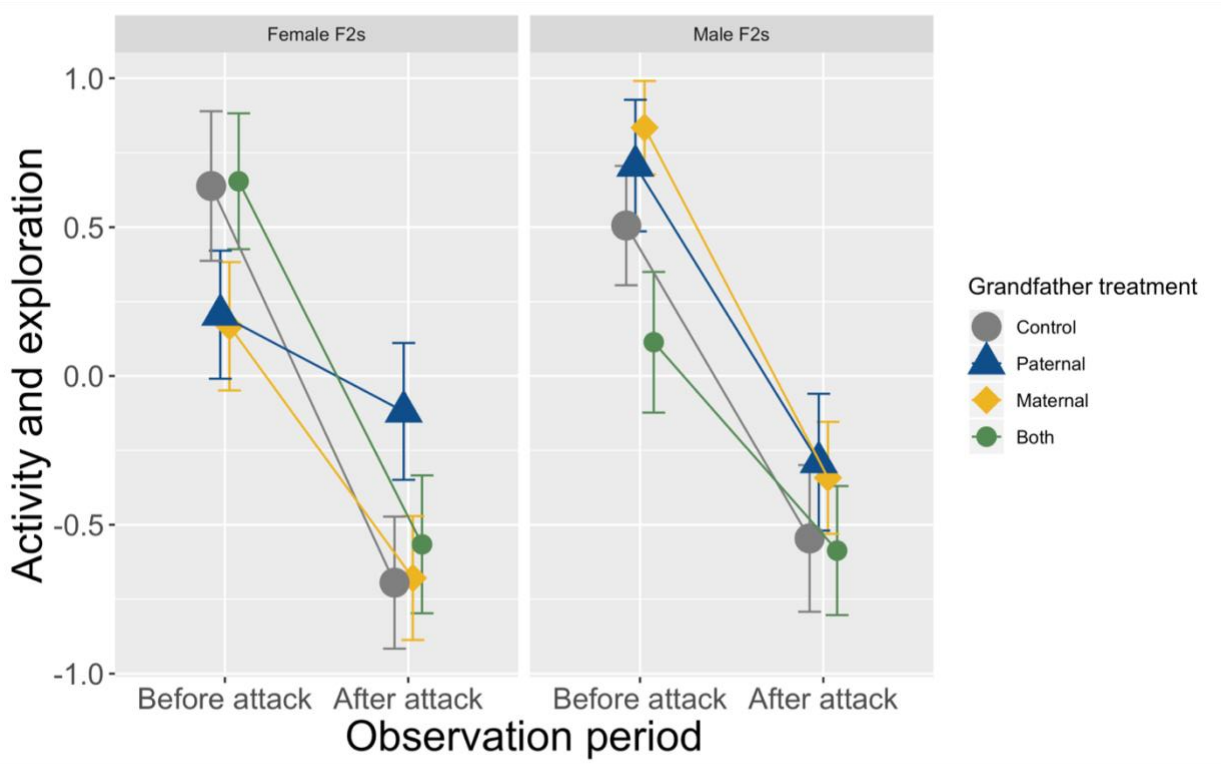

Figure 2: Activity and exploratory behavior (PCA: higher values are more active and exploratory individuals) of female (left; n=126) and male (right; n=125) F2s before and after the simulated predator attack in the open field assay (means with standard error bars). Female F2s with a predator-exposed paternal grandfather (blue) showed a reduced change in activity/exploration compared to female F2s with control grandfathers (grey), but there was no significant difference between female F2s with control grandfathers and female F2s with a predator-exposed maternal grandfather (yellow) or two predator-exposed grandfathers (green).

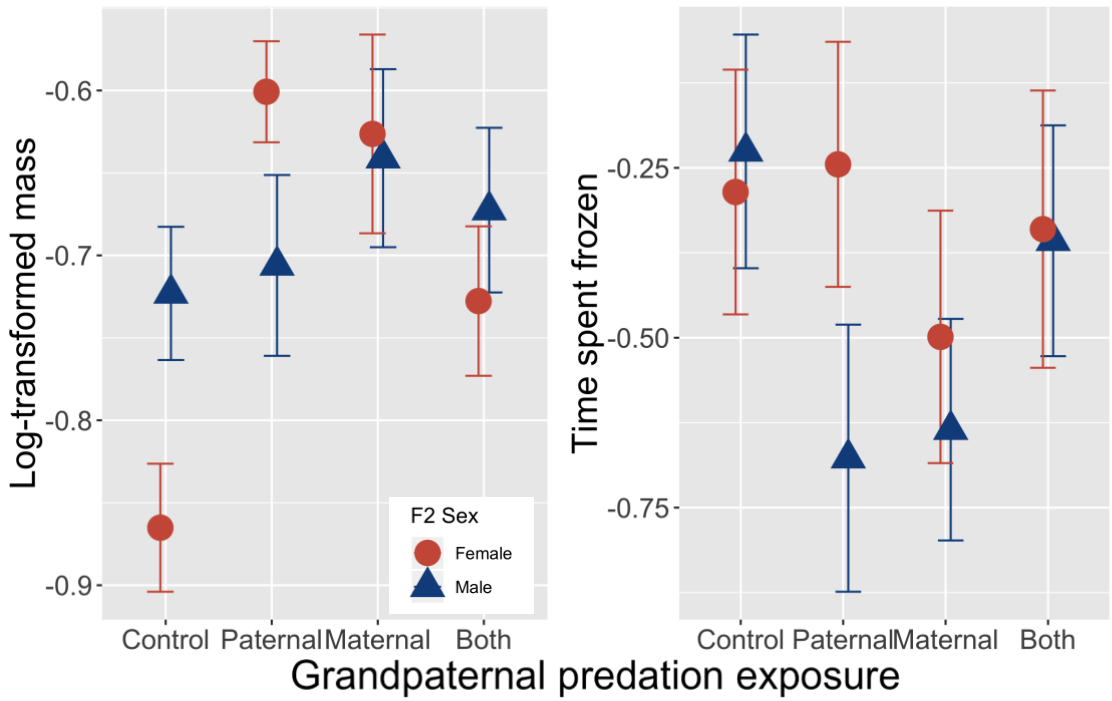

Figure 3: A) Mass (log-transformed) of female (red, circles) and male (blue, triangles) F2s with control grandfathers, predator-exposed paternal grandfather, predator-exposed maternal grandfather, or two predator-exposed grandfathers (5 months post-hatching, mean ± s.e.; n=125 males, n=126 females). B) Freezing behavior of female and male F2s across the four grandpaternal treatments (mean ± s.e.; n=125 males, n=126 females). Plotted on the y-axis are the residuals of the regression model, with grandparental treatment and sex removed.