TRANS-GENERATIONAL PLASTICITY

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TRANS-GENERATIONAL PLASTICITY

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Table of Contents

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Plant-Induced Transgenerational Plasticity Affecting Performance but Not Preference in a Polyphagous Moth

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Environmental variation experienced by a single genotype can induce phenotypic plasticity in various traits, such as behavioural, physiological and developmental characteristics. It can occur within the lifetime of an individual through within-generation phenotypic plasticity (WGP) or vertically across generations through transgenerational phenotypic plasticity (TGP). However, knowledge about TGP and the co-occurrence of WGP and TGP is still limited. In insect host-plant selection, the ability to alter phenotypic traits through WGP is well documented while the importance of TGP and the possible co-occurrence between the two is largely unknown. Host-plant selection of both larvae and adults of the polyphagous moth *Spodoptera littoralis* can be modified by previous experience through WGP. Thus, the aim of this study was to investigate if parental host-plant experience can influence host-plant choice behaviour and performance of *S. littoralis* offspring through TGP. For this, we tested effects of rearing parents on different host plants on the offspring's first instar larval migration and host plant choice, larval development and adult oviposition. A transgenerational effect on larval development was found, with increased pupal weight on a matching host-plant diet to that of the parent, when larvae were reared on cotton (good larval host plant) while no such effect was found on maize (poor larval host plant). These findings indicate that TGP of *S. littoralis* progeny development traits may only occur under favourable conditions. Parental diet did not affect larval host plant choice or migration. Furthermore, no effect of parental diet was found on offspring oviposition behaviour, indicating that adult female host-plant selection is governed by innate preference hierarchy and WGP, rather than TGP. Thus, parental diet may influence offspring performance but not behaviour, indicating that WGP is most important for host-plant selection behaviours in *S. littoralis*, but TGP can affect progeny development. If so, the importance of different types of plasticity may vary among traits of *S. littoralis* associated with host plant utilisation.

Keywords: within-generation phenotypic plasticity, transgenerational phenotypic plasticity, anticipatory plasticity, larval performance, insect behaviour, Lepidoptera, *Spodoptera littoralis*

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INTRODUCTION

Phenotypic plasticity is the ability of individual genotypes to modify traits, such as physiological, morphological and behavioural characteristics, quickly in response to biotic and abiotic environmental variation (West-Eberhard, 1989; Agrawal, 2001a; Whitman and Agrawal, 2009). It can increase the fitness of individuals in their experienced environments (Lande, 2009), and may include changes in both behaviour and development within the lifetime of an organism. Such within-generation plasticity (WGP) has been observed in diverse taxa, and theoretical models of the phenomenon have been widely supported with empirical data (West-Eberhard, 1989; Lande, 2009). Furthermore, phenotypic traits can be transferred vertically across generations through transgenerational plasticity (TGP), a non-genetic process that has been described in various ways, e.g., as parental effects, maternal effects, paternal effects, non-genetic inheritance, epigenetic inheritance, and prenatal learning (Mousseau and Fox, 1998; Uller, 2008; Bonduriansky et al., 2012; Peralta Quesada and Schausberger, 2012). TGP has been documented in plants (Herman and Sultan, 2011), vertebrates (Salinas and Munch, 2012), and invertebrates (Mousseau and Fox, 1998). However, there is still much less evidence of TGP than WGP, and theoretical models of TGP are not well supported by empirical data. Thus, there are substantial gaps in knowledge of TGP's roles and importance (Bonduriansky et al., 2012). Some empirical support for TGP in morphological and physiological traits has been reported (Herman and Sultan, 2011; Donelson et al., 2018; Yin et al., 2019). For example, herbivory of *Raphanus raphanistrum* plants may increase their offspring's leaf trichome density (Agrawal et al., 1999; Agrawal, 2001b) and changes in defensive features, earlier maturation and increased reproductive output have been observed in progeny of *Daphnia* parents exposed to predator cues (Agrawal et al., 1999; Walsh et al., 2015). Effects of parental experience on offspring behaviour have also been found, for example, fear conditioning in mice (Dias and Ressler, 2014) and feeding preferences of predatory mites (Peralta Quesada and Schausberger, 2012). However, although behaviour is considered to show high phenotypic plasticity, and organisms' behavioural traits are often the first to change in response to environmental changes (West-Eberhard, 1989), effects of TGP on behaviour have received less attention.

Predictive models have indicated that both WGP and TGP are favoured by spatial and temporal environmental heterogeneity, environmental cues that are reliable predictors of upcoming environmental conditions, and low costs of plasticity (Uller, 2008; Bonduriansky et al., 2012; Dury and Wade, 2019). However, there are differences in the theoretical frameworks regarding the kinds of conditions that favour WGP, TGP and their possible co-occurrence. Studies on *Daphnia spp.* have supported the decoupling of WGP and TGP, indicating that selective pressures tend to favour either one or the other (Walsh et al., 2015, 2016). It has also been argued that if either WGP or TGP can optimise a trait there is no need for a combination of the two (Donelson et al., 2018). Contrarily, other models have suggested that WGP and TGP can co-exist and that information from environmental cues can be integrated for a specific phenotypic trait (Leimar and McNamara, 2015). Empirical support for this theory has been provided, e.g., by indications of their co-occurrence in *Daphnia* defence mechanisms and development (Agrawal et al., 1999; Mikulski and Pijanowska, 2010) and drought adaptations of *Polygonium persicaria* (Sultan et al., 2009). Furthermore, it has been suggested that species with high ability to express WGP should also have high ability to express TGP (Woestmann and Saastamoinen, 2016), thus potentially favouring their co-occurrence.

Behavioural changes in foraging and host-finding induced by WGP have been well documented in various insects, including parasitoids (Turlings et al., 1993), honeybees (Menzel and Müller, 1996) and herbivores (Anderson and Anton, 2014). There is also evidence of insects' morphological and physiological traits being altered by TGP (Woestmann and Saastamoinen, 2016; Donelson et al., 2018). For example, parental exposure to UV light has been shown to affect wing coloration of offspring of the butterfly *Papilio polytes* (Katoh et al., 2018). Moreover, feeding on host plants of similar quality to plants their parents fed on has been found to promote development of offspring of both *Coenonympha pamphilus* and *Pieris rapae* (Cahenzli and Erhardt, 2013; Cahenzli et al., 2015). In addition, a study on the moth *Bicyclus anynana* showed that offspring preference for a synthetic odour was increased if the parents were reared on plant material coated with high doses of the same odour (Gowri et al., 2019). However, studies on effects of TGP on behaviour, particularly host-plant choice, using natural plant material are still lacking.

The Egyptian cotton leaf worm, *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae), is a generalist phytophagous insect (Pogue, 2002), with an innate preference hierarchy for host plants that can be modified by WGP in both larval and adult stages. Larval host plant feeding experience has been found to induce a preference for the experienced host plant in later larval stages (Carlsson et al., 1999), and subsequent adult moth oviposition and mating behaviour (Anderson et al., 2013; Thöming et al., 2013; Proffit et al., 2015). Moreover, mating experience affects subsequent reproductive behaviour of both male and female adults (Proffit et al., 2015). As the importance of WGP for host plant choice in *S. littoralis* is well established it provides a good model to investigate the occurrence of TGP and possible interactions between, and co-occurrence of, WGP and TGP. Thus, the objective of this study was to investigate whether TGP, induced by parental experience to host plants, can affect preferences and performance of the species' offspring. First, we investigated whether first instar larval host-plant choice and migratory behaviour are influenced by parental diet. We then followed the performance of larvae reared on the parental host plant or a different plant in a cross-comparison experiment. Finally, we tested whether the oviposition preference of the offspring was influenced by the parental diet.

MATERIALS AND METHODS

Plants and Insects

Plants of three species – cotton (*Gossypium hirsutum*, cv. Delta Pineland 90, Malvaceae), cowpea (*Vigna unguiculata* subsp.

Unguiculata, Fabaceae) and maize (*Zea mays*, cv. Sweet Nugget, Poaceae) – were used in this study. They were cultivated until use (before flowering) in experiments in a commercial substrate (Kronmull, Weibull Trädgård AB, Hammenhög, Sweden) in 1.5 L pots for 5–6 weeks at 25 ± 2°C, 70 ± 5% RH in a greenhouse with artificial light provided by Osram Powerstar HQI-T, 400 W/D lamps in 16:8 h L:D cycles.

The rearing strain of *S. littoralis* was founded from moths collected in Alexandria, Egypt, in 2008 and has been regularly refreshed with new wild-collected specimens from Egypt. Larvae were fed a potato-based artificial diet (Hinks and Byers, 1976) and kept at 25 ± 2°C, RH 65 ± 2% with 17:7 h L:D cycles. Adults were kept at 25 ± 2°C, RH 50 ± 2% with 16:8 h L:D cycles. Adult males and females were separated at the pupal stage and kept separate until mating.

Adult Oviposition Preference Rearing Procedure

The hypothesis that parental experience of this moth may affect oviposition preference of the offspring was tested in a four-generation rearing experiment, as follows. First-generation (F0) insects were reared on the artificial diet. Resulting pupae were sexed and kept separate. Sugar solution was provided as an energy source for the merging adults. Two to three days after hatching, single couples were mated in the absence of plants (F0). Mating of all replicates was observed and directly after mating the females were introduced to a cage with cotton plants to lay eggs. The offspring was then fed on cotton and adult females were introduced to a cage with cotton plants to mate and lay eggs. This was repeated for three generations (F1–F3). Females of the fourth generation were introduced to either cages with cotton plants or in the absence of plants for mating and oviposition, creating two separate rearing lines. One, consisting of larvae from eggs laid on cotton, was kept on cotton for the fourth generation (designated F4 cotton) while larvae of the other line, from eggs laid in cages with no plants, were kept on artificial diet (F4 artificial diet).

Adult Oviposition Preference

In this experiment, the females were allowed to choose to lay eggs on either cotton or cowpea plants. Larval experience of feeding on cotton has been previously shown to induce a preference for cotton over the innately preferred cowpea (Thöming et al., 2013). Females that had not been exposed to plant odours as adults of F0, F1, F2, F3, F4 generations fed on cotton and F4 fed on artificial diet were mated with unexposed males from the same feeding background and put in mating cages (length and width 28 cm; height 29 cm, $N = 25$ per treatment) containing detached cotton and cowpea leaves in water-filled vials (diameter 2 cm, height 9 cm). Detached leaves were used because they have previously given comparable results to intact plants in preference experiments (Thöming et al., 2013). Females were left in the cages and were able to oviposit on the leaves for 3 days. Cages were checked for eggs on a daily basis and eggs oviposited on the plants were removed and weighed.

Rearing Procedure for Larval Behaviour and Performance Assays

Eggs produced by the parental generation (F0) reared on artificial diet were collected and placed in plastic boxes (width 24 cm, height 7 cm, depth 18 cm) until hatching. Hatched larvae were randomly divided into two groups, one of which was fed on detached maize leaves and the other on detached cotton leaves. The plants were chosen partly because third and fourth instar larvae have different innate preferences for them, and partly because they differ in suitability as hosts, with cotton being considered a good host and maize a poor host (Anderson et al. unpublished data). Larvae were reared in groups of 60 individuals. Males and females were separated at the pupal stage, then after emergence males and females were transferred to a mating cage where mating occurred in the presence of the larval host plant. Eggs deposited on the plants were then removed and left to hatch in plastic boxes with no plant material.

Larval Host-Plant Choice Assay

Naïve first instar larvae (F1), from parents reared on either cotton ($N = 318$) or maize ($N = 326$), were placed in Petri dishes (diameter 8.5 cm, height 1.5 cm), each containing a maize leaf disc and a cotton leaf disc (both 0.5 cm diameter). Leaf discs were placed 5 cm apart in diametric opposition. To avoid positional effects, alternate replicates were rotated at 180° with respect to the others, so the leaf discs had different orientations. Since *S. littoralis* is known to use olfactory cues for host-plant identification (Salloum et al., 2011), the first choice was noted when a larva had oriented towards one of the leaf discs and touched it. Twenty-four batches of thirty randomly selected larvae were used in the tests, to ensure that the behaviour could be successfully observed. Larvae that did not make a choice within 4 h were excluded from further analysis.

Migration Assay

Naïve first instar larvae (F1) from parents reared on either cotton or maize were put in boxes (length 24 cm, width 18 cm, height 7 cm) containing cotton and maize leaves placed in water-filled vials (diameter 2 cm, height 9 cm). The larvae were put on either the plant that their parent was reared on or the other one. This resulted in four possible combinations, designated cotton × cotton, maize × maize, maize × cotton and cotton × maize, where the first and second plants are those that the parents and offspring were reared on, respectively. There were 20 replicates of each combination except cotton-maize (19) and 20 larvae in each replicate. To avoid positional effects, alternate replicates were rotated at 180° with respect to the others, so the leaves had different orientations. After 72 h, the number of larvae present on each plant was counted. Larvae that were not present on any of the plants were excluded from the experiment.

Performance Assay

Individual first instar larvae (F1), from parents fed on cotton or maize, were transferred to individual plastic cups (30 ml) containing either cotton or maize, creating four possible parent-offspring combinations (maize × maize, maize × cotton, cotton × maize, cotton × cotton: $N = 80$ in each case). Food

was provided *ad libitum* during their entire development, and their mortality, larval development time (period from hatching to pupation) and pupal weight were recorded. The larvae were checked daily during the later larval instars to see if pupation had occurred, and pupae were weighed 24 h after pupation.

Statistics

A preference index, based on the total egg weight oviposited on the two plants by each female in the adult oviposition preference assay, was defined as follows:

$$\textit{Adult oviposition preference index} = \frac{(\textit{egg weight on cotton} - \textit{egg weight on cowpea})}{(\textit{total egg weight})}$$

The index ranges from 1 (absolute preference for cotton) to −1 (absolute preference for cowpea), with 0 indicating no preference. Larval migration was calculated as the percentage of larvae that migrated from one plant to the other:

$$\textit{Larval migration} = \frac{(\textit{migrated larvae})}{(\textit{total number of larvae})}$$

This variable ranges from 0 (no migration) to 1 (migration of all larvae).

The Wilcoxon signed rank test was used to test the significance of between-treatment differences in non-parametric datasets, such as the female oviposition preference, larval migration and development time values (which did not satisfy the normal distribution null hypothesis of the Shapiro-Wilk test, at $P = 0.009$, $P < 0.001$ and $P = 0.003$, respectively). *Post hoc* pairwise comparisons were conducted using Dunn's test as implemented in the R package dunn.test (Dinno, 2017), with Bonferroni correction for the larval migration and development time. In the female oviposition bioassay, the response of the first generation (F0) was compared to the response of every other generation (F1–F4). To compensate for mass-significance, the *P*-values were multiplied by the number of relevant comparisons. A chi-square test was performed to assess the significance of preference differences in the larval host plant choice bioassay.

As pupal weight data obtained in the performance bioassay were not normally distributed (according to the Shapiro-Wilk test; $P < 0.001$) they were subjected to square-root transformation, then fitted using a linear model. Differences in pupal weight were analysed using ANOVA, with *post hoc* (Tukey's HSD) pairwise comparisons implemented using the *glht* function in the multicomp R package (Hothorn et al., 2008).

A chi-square test was applied to assess the significance of between-treatment differences in mortality rates in the performance bioassay. In all tests, $P < 0.05$ were considered statistically significant. All statistical analyses were performed using R statistical software version 3.6.1 (R Core Team, 2019), and figures were created with the software packages ggplot2 version 3.2.1 (Wickham, 2016) and ggpubr version 0.3.0 (Kassambara, 2020).

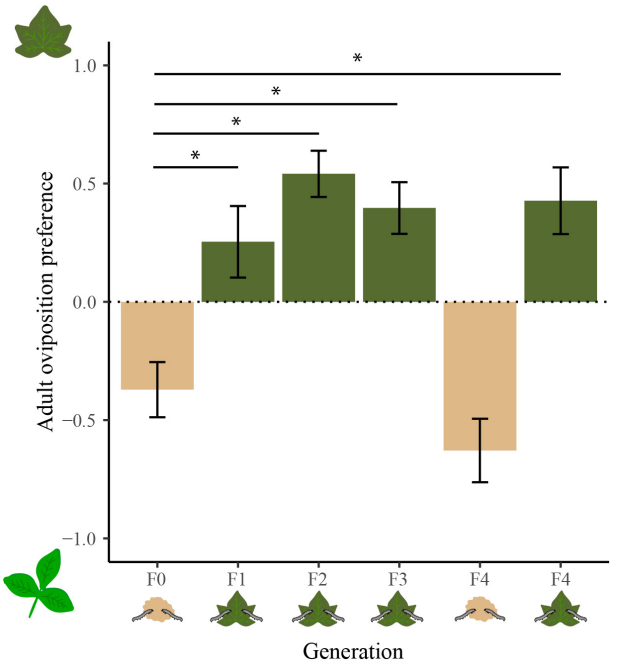

FIGURE 1 | Effects of larval diet experience on oviposition preference (mean ± SE of the preference index) of the five generations (F0–F4) reared on either the artificial diet (beige bars) or cotton (dark green bars) when given a choice of cotton (> 0) or cowpea (< 0) plants. Asterisks indicate significant difference between treatments (Dunn's test, $P < 0.05$).

RESULTS

Adult Oviposition Preference

We detected significant between-treatment differences in adults' host plant oviposition preferences (Wilcoxon signed rank test, $Z = -14.456$, $P < 0.001$; **Figure 1**). These included differences between the generation (F0) reared on the artificial diet and subsequent generations (F1–F4) reared on cotton ($N = 25$ in each case, Dunn's test: $Z = -2.884$, $P = 0.019$; $Z = -3.749$, $P < 0.001$; $Z = -2.950$, $P = 0.016$; and $Z = -3.470$, $P = 0.003$). In contrast, no difference in oviposition preference was found between the F0 and F4 generations reared on artificial diet ($N = 25$; $Z = 1.240$, $P = 1$).

Larval Host Plant Choice

Parental host plant experience had no significant effect on offspring host plant choice [$\chi^2(1) = 0.585$, $P = 0.444$; **Figure 2**]. Offspring from parents reared on cotton ($N = 318$) choose maize 41% and cotton 59% of the time while offspring from parents reared on maize ($N = 326$) choose maize 44% and cotton 56% of the time.

Larval Migration

We detected between-treatment differences in migratory behaviour of first instar larvae (Wilcoxon rank sum test, $Z = -6.782$, $P < 0.001$; **Figure 3**). Larvae placed on maize migrated more frequently [on average 73% migration in the

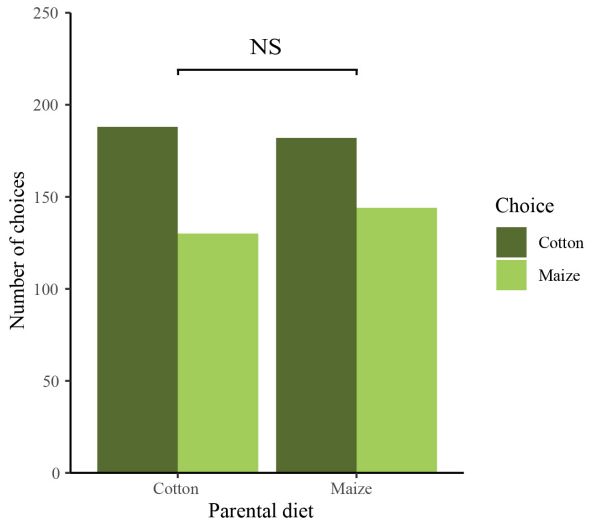

FIGURE 2 | Feeding preferences of first instar larvae: number of offspring that chose maize (light green bar) and cotton (dark green bar) of parents reared on cotton or maize. No significant effect of parental host plant experience was detected ($\chi^2 > 0.05$).

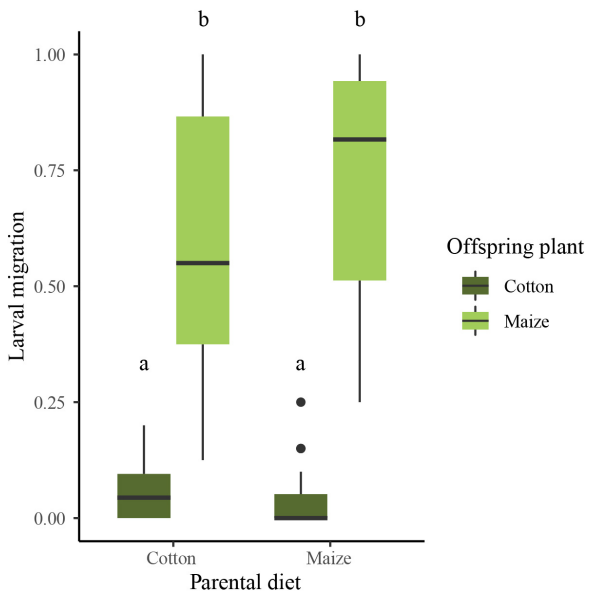

FIGURE 3 | Indices of migration from maize (light green boxes) and cotton (dark green boxes), ranging from 0 (no migration) to 1 (migration of all the larvae) of first instar larvae with matching and mismatching parental diets. Boxplots show means (black lines) and 25–75% percentiles. Whiskers show data ranges, excluding outliers (black circles, defined as values more than one box length from the upper and lower edges of the corresponding boxes). Different letters indicate significant between-treatment differences (Dunn's test, $P < 0.05$).

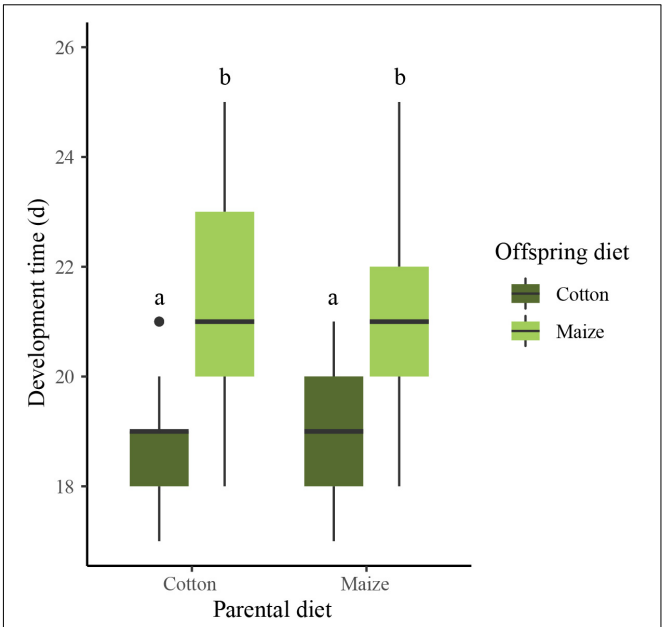

FIGURE 4 | Development time from first instar to pupation of larvae on maize (light green boxes) and cotton (dark green boxes), of parents reared on either a matching or mismatching diet. Boxplots show means (black lines) and 25–75% percentiles. Whiskers show data ranges, excluding outliers (black circles, defined as values more than one box length from the upper and lower edges of the corresponding boxes). Different letters indicate significant between-treatment differences (Dunn's test, $P < 0.05$).

maize × maize treatment ($N = 20$) and 59% migration in the cotton × maize treatment ($N = 20$)] than those placed on cotton [on average 4.5% migration in the maize × cotton treatment ($N = 20$) and 5% migration in the cotton × cotton treatment ($N = 20$)]. This applied to offspring of parents reared on both maize (maize × cotton versus maize × maize, Dunn's test, $Z = -5.783$, $P < 0.001$) and cotton (cotton × cotton versus cotton × maize, Dunn's test, $Z = -4.896$, $P < 0.001$). However, the parental diet had no effect on the migratory behaviour of offspring larvae (cotton × cotton versus maize × cotton, Dunn's test, $Z = 0.069$, $P = 1$; maize × maize versus cotton × maize, Dunn's test, $Z = -0.744$, $P = 1$).

Development Time

Development time from first larval instar to pupation differed between the treatments (Wilcoxon rank sum test, $Z = -21.6$, $P < 0.001$; **Figure 4**). Offspring on cotton developed faster than offspring on maize independently of parental diet [maize × cotton ($N = 79$) versus maize × maize ($N = 78$), Dunn's test, $Z = -7.154$, $P < 0.001$; cotton × cotton ($N = 77$) versus cotton × maize ($N = 76$), Dunn's test, $Z = -9.678$, $P < 0.001$]. The parental diet did not affect the development time when offspring were reared on cotton (cotton × cotton versus maize × cotton, Dunn's test, $Z = -1.884$, $P = 0.179$) or maize (maize × maize, cotton × maize, Dunn's test, $Z = 0.752$, $P = 1$).

Pupal Weight

Results of the larval performance bioassay showed that pupal weight differed between the treatments (LM, $F = 389$, $df = 3/306$, $P < 0.001$; **Figure 5**). Larvae reared on cotton had a higher pupal weight than larvae reared on maize, independently of

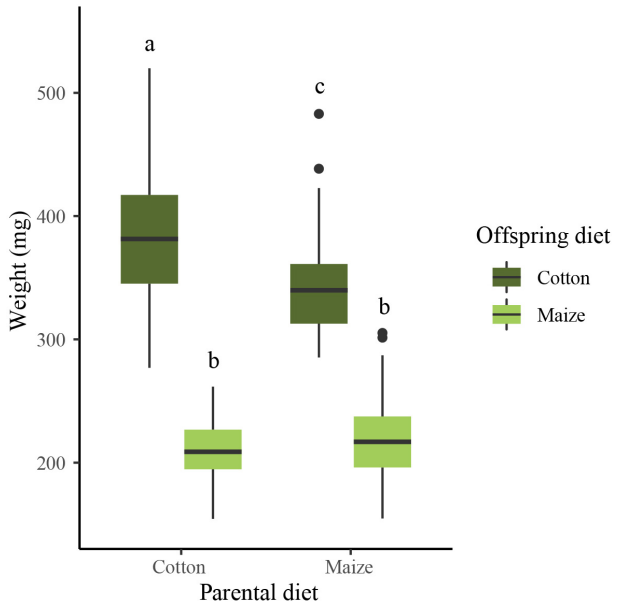

FIGURE 5 | Pupal weight of larvae reared on maize (light green boxes) and cotton (dark green boxes) of parents reared on either a matching or mismatching diet. Boxplots show means (black lines) and 25–75% percentiles. Whiskers show data ranges, excluding outliers (black circles, defined as values more than one box length from the upper and lower edges of the corresponding boxes). Different letters indicate significant between-treatment differences (LM, Tukey's HSD test, $P < 0.05$).

parental diet (maize × cotton versus maize × maize, $t = -20.925$, $P < 0.001$; cotton × cotton versus cotton × maize, $t = -26.889$, $P < 0.001$). Offspring reared on cotton with a matching parental diet had a higher pupal weight than offspring reared on cotton with mismatching parental diet (cotton × cotton versus maize × cotton, $t = -4.919$, $P < 0.001$). In contrast, no effect of parental diet on pupal weight was detected in offspring reared on maize (cotton × maize versus maize × maize, $t = 1.365$, $P = 0.523$).

Mortality

No difference in mortality rate was detected between the treatments: $\chi^2(3) = 2.06$, $P = 0.560$. Offspring reared on cotton with matching (cotton × cotton) and mismatching (maize × cotton) parental diets had mortality rates of 3.75 and 1.25%, respectively. Offspring reared on maize with matching (maize × maize) and mismatching (cotton × maize) parental diets had mortality rates of 2.5 and 5%, respectively.

DISCUSSION

In this study we found transgenerational effects of parental diet on larval development, but not the behaviour of progeny larvae or adults of *S. littoralis*. Larvae reared on cotton from parents reared on the same diet reached a higher pupal weight than larvae from parents reared on maize, but there was no difference in their development time. The difference observed between offspring reared on cotton with matching and mismatching parental diets could be explained by anticipatory transgenerational effects affecting offspring weight, e.g., through epigenetic modulations (Glastad et al., 2019) or vertically transferred symbionts (Paniagua Voirol et al., 2018). Increases in the fitness of offspring relative to parental fitness under matching conditions has been predicted in theoretical studies (Uller et al., 2013; Engqvist and Reinhold, 2016) and detected in both plants (Herman and Sultan, 2011) and vertebrates (Salinas and Munch, 2012). Anticipatory transgenerational effects have also been observed in invertebrates, including findings that offspring of the lepidopterans *Pieris rapae* and *Coenonympha pamphilus* developed best on food with the same nitrogen content as food that the parental generation had received (Rotem et al., 2003; Cahenzli and Erhardt, 2013). However, in our study a positive effect on offspring development was only found when offspring and parent diet was matched on the good host plant cotton, as no increase on offspring larval weight was found for offspring reared on maize with parents on a matching diet compared to the mismatching diet. When reared on a suboptimal host plant, the stressful environment limits the resources available for the parental generation and could reduce means of cue transfer through TGP to subsequent generations, thereby limiting the adaptive adjustment of the offspring (Uller et al., 2013). Effects of host plant quality have been observed in maritime pine, as Vivas et al. (2013) found that offspring of parents reared in benign conditions had higher pathogen resistance and growth rates than individuals grown in less favourable conditions. Furthermore, we have in *S. littoralis* found that on high quality food, larval olfactory experience is transferred to the adult through WGP and affect host plant choice while this does not occur on low quality food (Lhomme et al., 2018). Another possible explanation to the results could be silver spoon effects that allow parents from benign environments to give their offspring a heads start in life through transmission of abundant resources that would increase fitness independent of the environment of the offspring (Bonduriansky et al., 2012; Uller et al., 2013; Engqvist and Reinhold, 2016). In our study, we would expect that a silver spoon effect should increase the weight of offspring from parents reared on cotton irrespective of the larval diet, but we only found this effect on progeny that were fed cotton and not on those fed maize. Negative carry-over effects could potentially also be involved, we would then expect detrimental effects on the development of larvae with parents fed on maize. However, we detected no difference in pupal weight of offspring reared on maize related to the parental diet. Thus, we found no clear evidence for either silver spoon or carry-over effects.

The oviposition experiments revealed no transgenerational effects, as cotton versus cowpea preferences did not differ between females reared on the artificial diet after three generations on cotton and females of the first generation with no experience of plants during the larval stage. If transgenerational effects had influenced the oviposition preference, the females transferred to the artificial diet after three generations on cotton should have had a stronger preference for cotton than the first generation reared on the artificial diet. The oviposition results corroborate findings from our earlier studies that host plant selection of *S. littoralis* relies on an innate preference hierarchy

between host plants that is modified through WGP, where larval feeding experience induces a preference for the experienced plant (Thöming et al., 2013).

The migration assay clearly showed that there was a difference in the behaviour of the larvae on the two host plants. First instar larvae that were placed on maize migrated towards cotton at a much higher extent compared to the number of larvae migrating from cotton to maize. This is most likely due to that cotton is a more suitable host plant for larval development than maize (Anderson et al. unpublished data). The lower food quality of maize could induce larval movement and increase their search for an alternative host plant. A difference in migration behaviour has also been shown for larger larvae of *S. littoralis*, where more larvae were found to leave damaged cotton plants with induced defence than undamaged plants (Anderson et al., 2011). However, although *S. littoralis* larvae may actively migrate from less suitable plants, and WGP can strongly influence the species' feeding preference (Carlsson et al., 1999; Salloum et al., 2011), we detected no effect of parental diet on migration behaviour of first instar larvae, or larval host-plant choice.

Recent genetic and mathematical models of WGP, TGP and their possible coexistence predict that the two types of plasticity can operate either separately or additively, depending on the environmental conditions (Lande, 2009; Ezard et al., 2014; Leimar and McNamara, 2015; Dury and Wade, 2019). Empirical support for this has been found in both plants and animals (Agrawal et al., 1999; Sultan et al., 2009; Mikulski and Pijanowska, 2010; Walsh et al., 2015, 2016; Katoh et al., 2018). The experiments reported here provided no evidence of TGP affecting the behaviour of *S. littoralis*, supporting the hypothesis that the two types of plasticity operate separately for a specific trait, as postulated by Walsh et al. (2015, 2016). Theoretical models of WGP, TGP and their possible co-occurrence indicate that WGP is the dominating type of plasticity (Kuijper and Hoyle, 2015; Leimar and McNamara, 2015). However, the developmental data obtained in this study show that transgenerational effects may be present in *S. littoralis* when environmental conditions are favourable, and there may be interactions between WGP and TGP under these conditions. Thus, WGP and TGP could potentially influence specific traits connected to host plant utilisation either separately or additively.

WGP may influence behavioural choices of *S. littoralis* more strongly than TGP because the associated cues are temporally closer to them than parental cues, and more accurate predictors of current availabilities and qualities of host plants (Kuijper and Hoyle, 2015). We have previously identified a sensitive period in the late larval instar of *S. littoralis* in which larval host plant experience modifies subsequent adult behavioural decisions, while early larval experience is not retained (Lhomme et al., in press). Thus, the information is gathered close to the adult stage, and the decision mechanism takes into account factors that affect larval development. Such WGP increases the salience of the previously experienced plant, reduces risks of mismatching conditions in the ovipositing female's environment, and could make the transfer of parental experience through TGP redundant (Donelson et al., 2018). Furthermore, the host plant choice is under maternal control and she will likely lay her eggs on the same plant (through WGP), thereby reducing selective pressures favouring the evolution of TGP-mediated effects on the behaviour of offspring larvae. However, under such conditions, a transgenerational transfer of cues that increases the ability of the progeny to develop on that specific plant species could be very valuable and promote TGP of such traits.

In conclusion, this study suggests that TGP may modify the physiological state of *S. littoralis* offspring in a manner that enhances their performance on the parental host plant. However, such enhancement may only occur when conditions are favourable. The results also indicate that host plant-mediated behaviours of both adult females and first instar larvae are strongly influenced by innate preferences and WGP, but not by the parental diet. However, mechanisms underlying the higher pupal weight of offspring reared on cotton with a matching parental diet are still unknown, and further studies on anticipatory TGP, WGP and their possible co-occurrence in *S. littoralis* focused on these mechanisms in both favourable and unfavourable conditions are warranted.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AR, PA, PL, and MK designed the study. AR, PL, and MK collected and analysed the data. AR wrote the manuscript together with PL and PA. PA obtained the funding. All authors contributed to revisions.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Agrawal, A. A. (2001a). Phenotypic plasticity in the interactions and evolution of species. *Science* 294, 321–326. doi: 10.1126/science.1060701

Agrawal, A. A. (2001b). Transgenerational consequences of plant responses to herbivory: an adaptive maternal effect? *Am. Natural.* 157, 555–569. doi: 10.1086/319932

Agrawal, A. A., Laforsch, C., and Tollrian, R. (1999). Transgenerational induction of defences in animals and plants. *Nature* 401, 60–63. doi: 10.1038/43425

Anderson, P., and Anton, S. (2014). Experience-based modulation of behavioural responses to plant volatiles and other sensory cues in insect herbivores. *Plant Cell Environ.* 37, 1826–1835. doi: 10.1111/pce.12342

Anderson, P., Sadek, M. M., Larsson, M., Hansson, B. S., and Thöming, G. (2013). Larval host plant experience modulates both mate finding and oviposition choice in a moth. *Anim. Behav.* 85, 1169–1175. doi: 10.1016/j.anbehav.2013.03.002

Anderson, P., Sadek, M. M., and Wackers, F. L. (2011). Root herbivory affects oviposition and feeding behavior of a foliar herbivore. *Behav. Ecol.* 22, 1272–1277. doi: 10.1093/beheco/arr124

Bonduriansky, R., Crean, A. J., and Day, T. (2012). The implications of nongenetic inheritance for evolution in changing environments. *Evol. Appl.* 5, 192–201. doi: 10.1111/j.1752-4571.2011.00213.x

Cahenzli, F., and Erhardt, A. (2013). Transgenerational acclimatization in an herbivore-host plant relationship. *Proc. R. Soc. Lond. B Biol. Sci.* 280:2856. doi: 10.1098/rspb.2012.2856

Cahenzli, F., Wenk, B. A., and Erhardt, A. (2015). Female butterflies adapt and allocate their progeny to the host-plant quality of their own larval experience. *Ecology* 96, 1966–1973. doi: 10.1890/14-1275.1

Carlsson, M. A., Anderson, P., Hartlieb, E., and Hansson, B. S. (1999). Experience-dependent modification of orientational response to olfactory cues in larvae of *Spodoptera littoralis*. *J. Chem. Ecol.* 25, 2445–2454. doi: 10.1023/A:1020865922827

Dias, B. G., and Ressler, K. J. (2014). Parental olfactory experience influences behavior and neural structure in subsequent generations. *Nat. Neurosci.* 17, 89–96. doi: 10.1038/nn.3594

Dinno, A. (2017). *Dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums*. Available online at: https://CRAN.R-project.org/package=dunn.test (accessed June 1, 2020).

Donelson, J. M., Salinas, S., Munday, P. L., and Shama, L. N. (2018). Transgenerational plasticity and climate change experiments: where do we go from here? *Glob. Chang. Biol.* 24, 13–34. doi: 10.1111/gcb.13903

Dury, G. J., and Wade, M. J. (2019). When mother knows best: a population genetic model of transgenerational versus intragenerational plasticity. *J. Evol. Biol.* 33, 127–137. doi: 10.1111/jeb.13545

Engqvist, L., and Reinhold, K. (2016). Adaptive trans-generational phenotypic plasticity and the lack of an experimental control in reciprocal match/mismatch experiments. *Methods Ecol. Evol.* 7, 1482–1488. doi: 10.1111/2041-210x.12618

Ezard, T. H. G., Prizak, R., Hoyle, R. B., and Marshall, D. (2014). The fitness costs of adaptation via phenotypic plasticity and maternal effects. *Funct. Ecol.* 28, 693–701. doi: 10.1111/1365-2435.12207

Glastad, K. M., Hunt, B. G., and Goodisman, M. A. D. (2019). Epigenetics in insects: genome regulation and the generation of phenotypic diversity. *Annu. Rev. Entomol.* 64, 185–203. doi: 10.1146/annurev-ento-011118-111914

Gowri, V., Dion, E., Viswanath, A., Piel, F. M., and Monteiro, A. (2019). Transgenerational inheritance of learned preferences for novel host plant odors in *Bicyclus anynana* butterflies. *Evolution* 73, 2401–2414. doi: 10.1111/evo.13861

Herman, J. J., and Sultan, S. E. (2011). Adaptive transgenerational plasticity in plants: case studies, mechanisms, and implications for natural populations. *Front. Plant Sci.* 2:102. doi: 10.3389/fpls.2011.00102

Hinks, C., and Byers, J. (1976). Biosystematics of the genus Euxoa (Lepidoptera: Noctuidae): V. rearing procedures, and life cycles of 36 species. *Can. Entomol.* 108, 1345–1357. doi: 10.4039/Ent1081345-12

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometr. J.* 50, 346–363. doi: 10.1002/bimj.200810425

Kassambara, A. (2020). *ggpubr: 'ggplot2' Based Publication Ready Plots*. Available online at: https://rpkgs.datanovia.com/ggpubr/ (accessed June 1, 2020).

Katoh, M., Tatsuta, H., and Tsuji, K. (2018). Ultraviolet exposure has an epigenetic effect on a Batesian mimetic trait in the butterfly *Papilio polytes*. *Sci. Rep.* 8:31738. doi: 10.1038/s41598-018-31732-31738

Kuijper, B., and Hoyle, R. B. (2015). When to rely on maternal effects and when on phenotypic plasticity? *Evolution* 69, 950–968. doi: 10.1111/evo.12635

Lande, R. (2009). Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. *J. Evolut. Biol.* 22, 1435–1446. doi: 10.1111/j.1420-9101.2009.01754.x

Leimar, O., and McNamara, J. M. (2015). The evolution of transgenerational integration of information in heterogeneous environments. *Am. Natural.* 185, E55–E69. doi: 10.1086/679575

Lhomme, P., Carrasco, D., Larsson, M., Hansson, B., and Anderson, P. (2018). A context-dependent induction of natal habitat preference in a generalist herbivorous insect. *Behav. Ecol.* 29, 360–367. doi: 10.1093/beheco/arx173

Lhomme, P., Khallaf, M., Larsson, M., and Anderson P. (in press). Sensitive period for the induction of host plant preference in a generalist herbivorous insect. *Anim. Behav.*

Menzel, R., and Müller, U. (1996). Learning and memory in honeybees: from behavior to neural substrates. *Annu. Rev. Neurosci.* 19, 379–404. doi: 10.1146/annurev.ne.19.030196.002115

Mikulski, A., and Pijanowska, J. (2010). When and how can Daphnia prepare their offspring for the threat of predation? *Hydrobiologia* 643, 21–26. doi: 10.1007/s10750-010-0131-130

Mousseau, T. A., and Fox, C. W. (1998). *Maternal Effects AS Adaptations*. New York, NY: Oxford University Press.

Paniagua Voirol, L. R., Frago, E., Kaltenpoth, M., Hilker, M., and Fatouros, N. E. (2018). Bacterial symbionts in lepidoptera: their diversity, transmission, and impact on the host. *Front. Microbiol.* 9:556. doi: 10.3389/fmicb.2018.00556

Peralta Quesada, P. C., and Schausberger, P. (2012). Prenatal chemosensory learning by the predatory mite *Neoseiulus californicus*. *PLoS One* 7:e53229. doi: 10.1371/journal.pone.0053229

Pogue, M. G. (2002). A world revision of the genus Spodoptera Guenée:(Lepidoptera: Noctuidae). *Memoirs Am. Entomol. Soc.* 43, 1–202.

Proffit, M., Khallaf, M. A., Carrasco, D., Larsson, M. C., and Anderson, P. (2015). 'Do you remember the first time?' Host plant preference in a moth is modulated by experiences during larval feeding and adult mating. *Ecol. Lett.* 18, 365–374. doi: 10.1111/ele.12419

R Core Team (2019). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rotem, K., Agrawal, A. A., and Kott, L. (2003). Parental effects in *Pieris rapae* in response to variation in food quality: adaptive plasticity across generations? *Ecol. Entomol.* 28, 211–218. doi: 10.1046/j.1365-2311.2003.00507.x

Salinas, S., and Munch, S. B. (2012). Thermal legacies: transgenerational effects of temperature on growth in a vertebrate. *Ecol. Lett.* 15, 159–163. doi: 10.1111/j.1461-0248.2011.01721.x

Salloum, A., Colson, V., and Marion-Poll, F. (2011). Appetitive and Aversive Learning in Spodoptera littoralis Larvae. *Chem. Senses* 36, 725–731. doi: 10.1093/chemse/bjr041

Sultan, S. E., Barton, K., and Wilczek, A. M. (2009). Contrasting patterns of transgenerational plasticity in ecologically distinct congeners. *Ecology* 90, 1831–1839. doi: 10.1890/08-1064.1

Thöming, G., Larsson, M. C., Hansson, B. S., and Anderson, P. (2013). Comparison of plant preference hierarchies of male and female moths and the impact of larval rearing hosts. *Ecology* 94, 1744–1752. doi: 10.1890/12-0907.1

Turlings, T. C. L., Wäckers, F. L., Vet, L. E. M., Lewis, W. J., and Tumlinson, J. H. (1993). "Learning of host-finding cues by hymenopterous parasitoids," in *Insect Learning: Ecological and Evolutionary Perspectives*, eds D. R. Papaj and A. C. Lewis (Boston, MA: Springer), 51–78. doi: 10.1007/978-1-4615-2814-2_3

Uller, T. (2008). Developmental plasticity and the evolution of parental effects. *Trends Ecol. Evol.* 23, 432–438. doi: 10.1016/j.tree.2008.04.005

Uller, T., Nakagawa, S., and English, S. (2013). Weak evidence for anticipatory parental effects in plants and animals. *J. Evolut. Biol.* 26, 2161–2170. doi: 10.1111/jeb.12212

Vivas, M., Zas, R., Sampedro, L., and Solla, A. (2013). Environmental maternal effects mediate the resistance of maritime pine to biotic stress. *PLoS One* 8:e70148. doi: 10.1371/journal.pone.0070148

Walsh, M. R., Castoe, T., Holmes, J., Packer, M., Biles, K., Walsh, M., et al. (2016). Local adaptation in transgenerational responses to predators. *Proc. R. Soc. B Biol. Sci.* 283:2271. doi: 10.1098/rspb.2015.2271

Walsh, M. R., Cooley, F., Biles, K., and Munch, S. B. (2015). Predator-induced phenotypic plasticity within-and across-generations: a challenge for theory? *Proc. R. Soc. B Biol. Sci.* 282:2205. doi: 10.1098/rspb.2014.2205

West-Eberhard, M. J. (1989). Phenotypic plasticity and the origins of diversity. *Annu. Rev. Ecol. Syst.* 20, 249–278. doi: 10.1146/annurev.es.20.110189.001341

Whitman, D. W., and Agrawal, A. A. (2009). "What is phenotypic plasticity and why is it important," in *Phenotypic Plasticity Of Insects: Mechanisms And Consequences*, 1st Edn, ed. D. Whitman (Boca Raton: CRC Press), 1–63.

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag.

Woestmann, L., and Saastamoinen, M. (2016). The importance of trans-generational effects in *Lepidoptera*. *Curr. Zool.* 62, 489–499. doi: 10.1093/cz/zow029

Yin, J., Zhou, M., Lin, Z., Li, Q. Q., and Zhang, Y. Y. (2019). Transgenerational effects benefit offspring across diverse environments: a meta-analysis in plants and animals. *Ecol. Lett.* 22, 1976–1986. doi: 10.1111/ele.13373

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Corrigendum: Plant-Induced Transgenerational Plasticity Affecting Performance but Not Preference in a Polyphagous Moth

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In the original article, there was a mistake in **Figure 5** as published. On the y-axis, the weight of the pupae is given in gram (g) while it should be milligram (mg). The corrected **Figure 5** appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

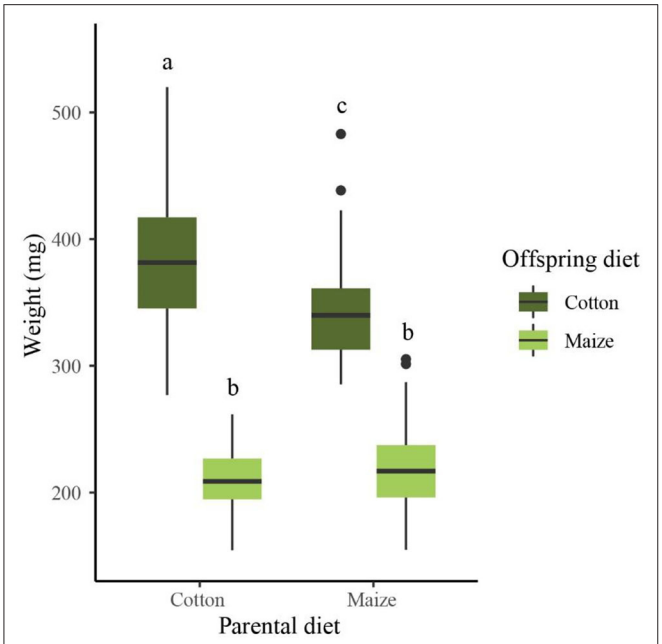

FIGURE 5 | Pupal weight of larvae reared on maize (light green boxes) and cotton (dark green boxes) of parents reared on either a matching or mismatching diet. Boxplots show means (black lines) and 25–75% percentiles. Whiskers show data ranges, excluding outliers (black circles, defined as values more than one box length from the upper and lower edges of the corresponding boxes). Different letters indicate significant between-treatment differences (LM, Tukey's HSD test, $P < 0.05$).

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Transgenerational Plasticity in the Context of Predator-Prey Interactions

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Almost all animal species are engaged in predator-prey interactions. These interactions, variable in time and space, favor the emergence and evolution of phenotypic plasticity, which allows prey to fine-tune their phenotype to the current risk of predation. A famous example is the induction of defensive neck-teeth, spines or helmets in some water fleas when they detect cues of predator presence. In general, the response may involve different types of traits (behavioral, morphological, physiological, and life-history traits), alone or in combination. The induced traits may be adaptive anti-predator defenses or reflect more general stress-based responses. Recently, it has been found that predator-induced plasticity occurs not only within but also across generations (transgenerational plasticity), i.e., the phenotype of a generation is influenced by the detection of predator-cues in previous generation(s), even if the current generation is not itself exposed to these cues. In this paper, we aim to review this accumulating literature and propose a current state of key aspects of predator-induced transgenerational plasticity in metazoans. In particular, we review whether patterns of predator-induced transgenerational plasticity depend on the type of traits. We analyze the adaptive value of predator-induced transgenerational plasticity and explore the evidence for its evolution and underlying mechanisms. We also consider its temporal dynamics: what are the time windows during which predator-cues must be detected to be transmitted across generations? Are transgenerational responses in offspring stage-dependent? How many generations does transgenerational plasticity persist? Finally, we discuss other factors highlighted in the literature that influence predator-induced transgenerational plasticity: what are the relative contributions of maternal and paternal exposure to predator-cues in generating transgenerational plasticity? Do transgenerational responses depend on offspring sex? Do they scale with the perceived level of predation risk? This review shows that we are only at the beginning of understanding the processes of predator-induced transgenerational plasticity, and it encourages future research to fill the lack of knowledge highlighted here.

Keywords: non-genetic inheritance, inducible defense, intergenerational plasticity, parental effects, sex dependence, sensitive windows

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INTRODUCTION

Research on predator-prey interactions and phenotypic plasticity has been intimately linked for a long time, merging ecological and evolutionary considerations. At the beginning of the 20th century, the mutation theory of de Vries and MacDougal (1905) was predominant, stating that organismal characters change because of relatively rare and heritable changes in the genetic material. Stimulated by the short-comings of the mutation theory, Richard Woltereck was the first to conceptualize that the phenotype is also influenced by the environment (Woltereck, 1909). Using *Daphnia* water fleas as a model system, he experimentally demonstrated that morphological shape (the relative size of the head) varied in relation to different nutrient levels (Nicoglou, 2018). He drew "phenotypic curves" to describe the phenomenon and coined the term reaction norm ("Reaktionsnorm"). Using experimental approaches on a model that is easy and cheap to raise, he thus paved the way for future studies on phenotypic plasticity—now generally defined as the ability of a given genotype to express alternative phenotypes under different environmental conditions (Pigliucci, 2005; namely within-generational plasticity, WGP hereafter). Particularly, his findings inspired studies on plastic responses in predator-prey systems, of which morphological change of *Daphnia* in presence of predators is the most famous example (Riessen, 1999; Tollrian and Harvell, 1999). A rich literature [starting with Gilbert's (1966) study on rotifers and Jacobs (1967) on daphnids] has demonstrated predator-induced defenses in prey (e.g., mammals: Hunter and Skinner, 1998; amphibians: Van Buskirk, 2002; insects: Li and Lee, 2004; birds: Eggers et al., 2006; mollusks: Auld and Relyea, 2011; actinopterygii: Torres-Dowdall et al., 2012; reviewed by Riessen, 1999; Tollrian and Harvell, 1999) as well as, to a lesser degree, prey-induced offenses in predators (e.g., Padilla, 2001; Kopp and Tollrian, 2003; Kishida et al., 2010). Theory suggests that the evolution of phenotypic plasticity is favored by the highly variable nature of predator-prey interactions in both time and space (e.g., Lima, 1998; Sih et al., 2000; Svanbäck et al., 2009). In this review, we will focus on predator-induced plasticity in prey, so-called inducible defenses, which remains the most common case of phenotypic plasticity in predator-prey systems (Tollrian and Harvell, 1999). These anti-predator responses are triggered when prey detect specific (mechanical, visual, auditory, chemical) cues of predator presence in their environment—chemical cues being the most common (predator-specific odor, dietary cues or alarm cues from injured prey; Mitchell et al., 2017). The response to predator-cues can involve different types of traits (behavioral, morphological, physiological and life-history traits), alone or in combination. These traits can confer defensive value or reflect stress-based responses. The optimal defense strategies depend on the relative speed between the expression of traits and both the onset and duration of risk (Steiner and Pfeiffer, 2007; Mitchell et al., 2017).

Ninety years after the presentation of R. Woltereck at the German Society of Zoology, and after a rich literature on inducible defenses in water fleas had accumulated, Agrawal et al. (1999) showed that *Daphnia cucullata* exposed to cues from dipteran larvae not only express morphological defenses, but also produce offspring that are better defended than those from unthreatened parents. This study pioneered the general idea that phenotypic plasticity can occur across generations and that such transgenerational plasticity (hereafter TGP) may play an important role in predator-prey interactions (Agrawal et al., 1999). Since then, a growing number of studies has examined the ecological and evolutionary importance of TGP both in general (**Figure 1A**) and in the context of predator-prey interactions (**Figure 1B**). In the present review, we aim to propose a synthesis of the key aspects of predator-induced TGP in metazoans. We define TGP as all phenotypic changes in a new generation that are triggered by the environment experienced by the previous generation(s). This broad definition (also used in very recent reviews: Bell and Hellmann, 2019; Yin et al., 2019; Donelan et al., 2020) allows to encompass effects on offspring phenotype due to the effect of predator exposure on maternal or paternal conditions (state-based TGP) and due to signals transmitted by parents (adaptive TGP). To search for relevant studies, we used the following keywords in the Web Of Science Core collection: ("trans?generational plasticity" OR "trans?generational effect?" OR 'trans?generational response?" OR "inter?generational effect?" OR "inter?generational plasticity" OR "inter?generational response?" OR "maternal effect?" OR "paternal effect?" OR "parental effect?" OR "grand?parental effect?" OR "maternal programming" OR "paternal programming" OR "parental programming" OR "maternal care" OR "paternal care" OR "parental care" OR "maternal environment" OR "paternal environment" OR "parental environment" OR "grand?parental environment" OR "across generations") AND (anti?predator OR predation OR predator) and we filtered the results in the subject areas 'environmental sciences ecology' and 'evolutionary biology.' Our review includes (1) studies that do not explicitly use the term 'transgenerational plasticity' but that fit in our definition of TGP, (2) studies in which transgenerational and within-generational responses interact (non-additive effects; e.g., Agrawal et al., 1999; Beaty et al., 2016) or not (additive effects; e.g., Salinas et al., 2013; Luquet and Tariel, 2016), (3) studies involving adaptive (e.g., inducible defenses) and/or non-adaptive (e.g., state-based TGP) responses, and (4) studies in which parental exposure to predator-cues occurred after fertilization, but in which it seems unlikely that the early developmental stages of offspring were able to directly perceive predator-cues on their own (e.g., exposure to olfactory predator-cues of gravid females, visual predator-cues during parental care on eggs). However, we discarded (1) studies that focus on offspring traits without considering the eco-evolutionary framework of predator-prey interactions (e.g., cognitive abilities: Coutellier and Würbel, 2009; Roche et al., 2012; response to contaminants: Plautz et al., 2013), using predation risk only as a stressor, and (2) studies that focus only on the reproductive performance of mothers (e.g., egg size or clutch size) without evaluating later effects on offspring traits (e.g., Segers and Taborsky, 2012; Tigreros et al., 2019). Moreover, this review, specifically focused on TGP in the context of predator-prey interactions, benefits from the very recent reviews on TGP in general (Bell and Hellmann, 2019; Yin et al., 2019) and in other ecological contexts (climate

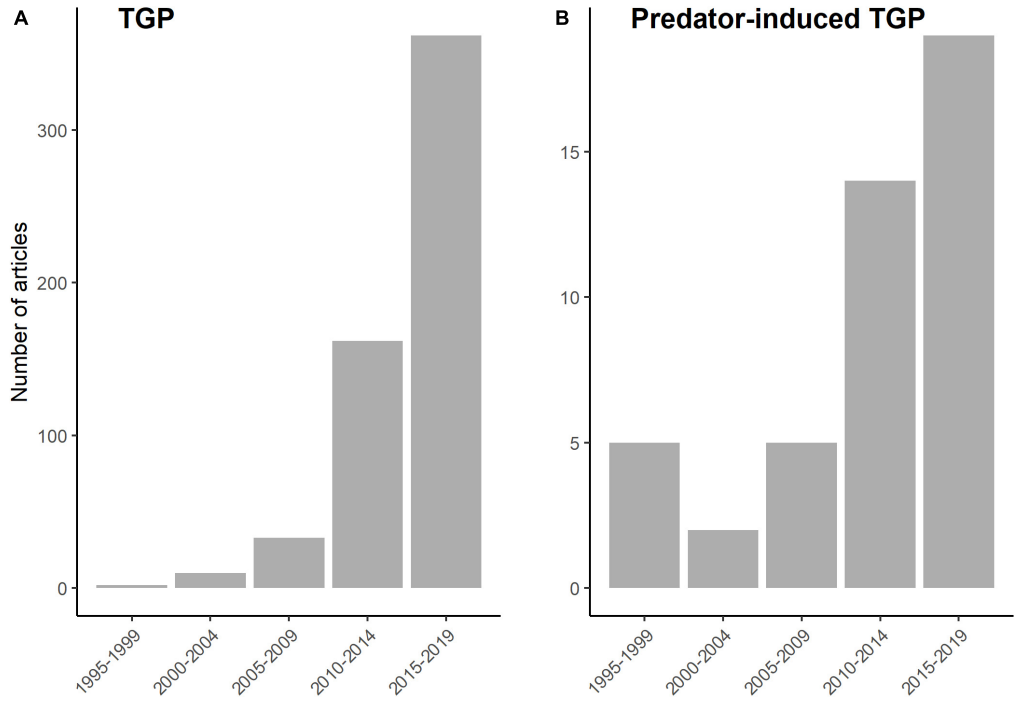

FIGURE 1 | Growing interest in (**A**) transgenerational plasticity and (**B**) in predator-induced transgenerational plasticity. **(A)** List of articles extracted from the Core Collection of Web of science using the keyword "transgenerational plasticity." **(B)** List of articles included in our review (**Table 1**), see the Introduction for the detailled selection procedure.

change: Donelson et al., 2018; human-altered environment: Donelan et al., 2020).

In total, we reviewed 55 empirical studies investigating predator-induced TGP using different predator signals (27 chemical, 17 visual, 5 auditory, 13 real predator) in various prey species (**Table 1**). Of these, 40 studies are empirical demonstrations of predator-induced TGP, while the other 15, mainly from the past 2 years, tested more specific hypotheses about TGP. TGP is therefore a growing research field, where the underlying processes are just starting to be explored. Our review is structured as follows: first, we summarize the types of traits involved in predator-induced TGP. Second, we focus on the evolutionary aspects of predator-induced TGP: what is its adaptive value? Can transgenerational responses evolve? What are the underlying mechanisms of inheritance? Third, we analyze the temporal dynamics of predator-induced TGP: what are the sensitive developmental windows in parents and offspring? How many generations does the influence of predators persist in prey? Fourth, we discuss other key aspects of predator-induced TGP: sex-specific patterns and adjustment to the intensity of predation.

TYPE OF TRAITS INVOLVED IN PREDATOR-INDUCED TRANSGENERATIONAL PLASTICITY

Different types of traits (morphological, behavioral, life-history, and physiological) are involved in within-generational responses to predation (Tollrian and Harvell, 1999). These same traits can be influenced in offspring by parental exposure to predator-cues (**Tables 2**, **3**), as for instance shell thickness in freshwater snails (Beaty et al., 2016; Luquet and Tariel, 2016), activity in sticklebacks (Stein et al., 2018; Hellmann et al., 2019), size at maturation in water fleas (Tollrian, 1995; Walsh et al., 2016) or corticosteroïds in hares (Sheriff et al., 2010). In the TGP studies we reviewed, 45% of traits are anti-predator defenses (e.g., longer helmet in *Daphnia*, crawling-out behavior in *Physa* freshwater snails), whereas the remaining 55% are more general responses (life-history: e.g., body size and mass; physiological stress-response: e.g., plasma cortisol; (epi)genomic modifications: e.g., brain gene expression; **Tables 2**, **3**). Some of these more general responses may nevertheless contribute to anti-predator strategy, such as larger eggs (McGhee et al., 2012) or higher size at birth of offspring prey (Donelan and Trussell, 2018b), which allow to faster reach a refuge-size. In other cases, the responses are more likely to be by-products of carry-over effects of parental exposure to predator-cues (stress-based responses, e.g., smaller egg size: McGhee et al., 2012; smaller size at birth: Monteforte et al., 2020) or reflect trade-offs between anti-predator defenses and other functions in offspring (e.g., induction of morphological defenses at the expense of growth rate or investment in reproduction: DeWitt et al., 1998). For example, two studies (Stein and Bell, 2014; Stein et al., 2018) showed that stickleback offspring produced by fathers exposed to predation risk were lighter and smaller. It is possible that these offspring received less fanning (oxygen) from predator-exposed fathers, altering growth

TABLE 1 | Relevant information of the 55 papers studying transgenerational plasticity in the context of predator-prey interactions.

References	Prey species	Taxonomy	Predator species	Offspring traits	Cues	Parental sex exposed	Number of generations concerned	Parental stage exposed	Offspring stage concerned
Agrawal et al., 1999	Water flea (*Daphnia cucullata*)	Branchiopoda	Phantom midge (*Chaoborus avican*)	Morphology	Real predator	Mother	3	Entire life	Neonates Adult
Basso et al., 2014	Great tit (*Parus major*)	Aves	Sparrowhawk (*Accipiter nisus*)	Morphology Life-history	Auditory	Mother	2	Adult	Neonates Larval/juvenile
Basso and Richner, 2015a	Great tit (*Parus major*)	Aves	Short-tailed weasel (*Mustela erminea*)	Morphology Life-history	Visual Auditory	Mother	2	Adult	Neonates Larval/juvenile
Basso and Richner, 2015b	Great tit (*Parus major*)	Aves	Short-tailed weasel (*Mustela erminea*)	Morphology Life-history	Visual Auditory	Mother	2	Adult	Neonates Larval/juvenile
Beaty et al., 2016	Freshwater snail (*Physa acuta*)	Gastropoda	Southern plains crayfish (*Procambarus simulans*)	Behavior Morphology	Chemical	Biparental	2	Larval/juvenile	Adult
Bestion et al., 2014	Common lizard (*Zootoca vivipara*)	Squamata	Green whip snake (*Hierophis viridiflavus*)	Behavior Life-history Morphology	Chemical	Mother	2	Adult	Neonates Larval/juvenile
Coslovsky and Richner, 2011	Great tit (*Parus major*)	Aves	Eurasian sparrowhawk (*Accipiter nisus*)	Behavior Morphology Life-history	Visual Auditory	Mother	2	Adult	Neonates Larval/juvenile
Coslovsky and Richner, 2012	Great tit (*Parus major*)	Aves	Eurasian sparrowhawk (*Accipiter nisus*)	Life-history Morphology	Visual Auditory	Mother	2	Adult	Neonates Larval/juvenile
Dixon and Agarwala, 1999	Pea aphid (*Acyrthosiphon pisum*)	Insecta	Two-spot ladybird (*Adalia bipunctata*)	Morphology	Chemical	Mother	2	Adult	Adult
Donelan and Trussell, 2015	Marine snail (*Nucella lapillus*)	Gastropoda	Green shore crab (*Carcinus maenas*)	Behavior Physiology	Chemical	Biparental	2	Adult	Larval/juvenile
Donelan and Trussell, 2018a	Marine snail (*Nucella lapillus*)	Gastropoda	Green shore crab (*Carcinus maenas*)	Behavior Life-history Physiology	Chemical	Biparental	2	Adult	Larval/juvenile
Donelan and Trussell, 2018b	Marine snail (*Nucella lapillus*)	Gastropoda	Green shore crab (*Carcinus maenas*)	Life-history	Chemical	Biparental	2	Adult	Neonates
Dzialowski et al., 2003	Water flea (*Daphnia lumholtzi*)	Branchiopoda	Phantom midge (*Chaoborus punctipennis*) Bluegill sunfish (*Lepomis macrochirus*)	Morphology	Chemical	Mother	2	Larval/juvenile	Neonates
Evans et al., 2007	Guppy (*Poecilia reticulata*)	Actinopterygii	Northern pike (*Esox lucius*)	Behavior Morphology	Visual Chemical	Mother	2	Adult	Neonates
Freinschlag and Schausberger, 2016	Spider mite (*Tetranychus urticae*)	Arachnida	Predatory mite (*Phytoseiulus persimilis*)	Behavior Life-history	Real predator Chemical	Mother	2	Adult	Larval/juvenile

(Continued)

TABLE 1 | Continued

References	Prey species	Taxonomy	Predator species	Offspring traits	Cues	Parental sex exposed	Number of generations concerned	Parental stage exposed	Offspring stage concerned
Giesing et al., 2011	Three-spined stickleback (*Gasterosteus aculeatus*)	Actinopterygii	Northern pike (*Esox lucius*)	Behavior Life-history Physiology	Visual	Mother	2	Adult	Embryonic Larval/juvenile
Goeppner et al., 2020	Freshwater snail (*Physa acuta*)	Gastropoda	Bluegill sunfish (*Lepomis macrochirus*)	Morphology	Chemical	Biparental	2	Entire life	Adult
Hales et al., 2017	Water flea (*Daphnia ambigua*)	Branchiopoda	Redbreast sunfish (*Lepomis auritus*)	Genomic	Chemical	Biparental	3	Larval/juvenile	Adult
Hellmann et al., 2019	Three-spined stickleback (*Gasterosteus aculeatus*)	Actinopterygii	Prickly sculpin (*Cottus asper*)	Behavior Life-history Genomic	Visual	Mother Father Biparental	3	Adult	Larval/juvenile
Hellmann et al., 2020	Three-spined stickleback (*Gasterosteus aculeatus*)	Actinopterygii	Prickly sculpin (*Cottus asper*)	Behavior Life-history Physiology	Visual	Father	2	Adult	Larval/juvenile
Keiser and Mondor, 2013	Pea aphid (*Acyrthosiphon pisum*)	Insecta	Alarm pheromone ((*E*)-β-farnesene)	Behavior	Chemical	Mother	2	Larval/juvenile Adul	Larval/juvenile
Kunert and Weisser, 2003	Pea aphid (*Acyrthosiphon pisum*)	Insecta	Marmalade hoverfly (*Episyrphus balteatus*) Common green lacewing (*Chrysoperla carnea*)	Morphology	Real predator	Mother	2	Larval/juvenile Adult	Larval/juvenile Adult
Kunert and Weisser, 2005	Pea aphid (*Acyrthosiphon pisum*)	Insecta	Common green lacewing (*Chrysoperla carnea*)	Morphology	Real predator	Mother	2	Larval/juvenile Adult	Larval/juvenile Adult
Lehto and Tinghitella, 2019	Three-spined stickleback (*Gasterosteus aculeatus*)	Actinopterygii	Shorthead sculpin (*Cottus confusus*)	Physiology Life-history	Visual Physical	Mother Father Biparental	2	Adult	Adult
Li and Zhang, 2019	Spider mite (*Tetranychus urticae*)	Arachnida	Predatory mite (*Phytoseiulus persimilis*)	Life-history	Chemical	Mother	2	Larval/juvenile	Entire life
Luquet and Tariel, 2016	Freshwater snail (*Physa acuta*)	Gastropoda	Red swamp crayfish (*Procambarus clarkii*)	Behavior Morphology	Chemical	Biparental	2	Larval/juvenile Adult	Adult
McGhee and Bell, 2014	Three-spined stickleback (*Gasterosteus aculeatus*)	Actinopterygii	Northern pike (*Esox lucius*)	Behavior Genomic	Visual	Father	2	Adult	Larval/juvenile
McGhee et al., 2012	Three-spined stickleback (*Gasterosteus aculeatus*)	Actinopterygii	Northern pike (*Esox lucius*)	Behavior Life-history	Visual	Mother	2	Adult	Larval/juvenile
Mikulski and Pijanowska, 2010	Water flea (*Daphnia magna*)	Branchiopoda	Crucian carp (*Carassius carassius*)	Life-history	Chemical	Mother	2	Neonates Larval/juvenile	Adult

(Continued)

TABLE 1 | Continued

References	Prey species	Taxonomy	Predator species	Offspring traits	Cues	Parental sex exposed	Number of generations concerned	Parental stage exposed	Offspring stage concerned
Mommer and Bell, 2013	Three-spined stickleback (*Gasterosteus aculeatus*)	Actinopterygii	Northern pike (*Esox lucius*)	Physiology	Visual	Mother	2	Adult	Adult
Mondor et al., 2005	Cotton aphid (*Aphis gossypii*)	Insecta	Convergent lady beetle (*Hippodamia convergens*)	Morphology	Chemical	Biparental	2	Adult	Adult
Monteforte et al., 2020	Guppy (*Poecilia reticulata*)	Actinopterygii	American cichlid (*Crenicichla alta*)	Life-history Genomic	Visual Chemical	Mother	2	Adult	Neonates
Morales et al., 2018	Yellow-legged gull (*Larus michahellis*)	Aves	American mink (*Neovison vison*)	Behavior Life-history	Visual	Mother	2	Adult	Neonates
Podjasek et al., 2005	Pea aphid (*Acyrthosiphon pisum*)	Insecta	Alarm pheromone (*E*)-β-farnesene	Morphology	Chemical	Mother	2	Larval/juvenile Adult	Adult
Schield et al., 2016	Water flea (*Daphnia ambigua*)	Branchiopoda	Redbreast sunfish (*Lepomis auritus*)	Genomic	Chemical	Biparental	2	Entire life	Neonates
Segers et al., 2011	Mouthbrooding cichlid (*Eretmodus cyanostictu*)	Actinopterygii	African cichlid (*Ctenochromis horei*)	Life-history	Visual	Mother	2	Adult	Larval/juvenile
Seiter and Schausberger, 2015	Predatory mite (*Phytoseiulus persimilis*)	Arachnida	Predatory mite (*Amblyseius andersoni*)	Behavior	Real predator	Mother	2	Adult	Larval/juvenile
Sentis et al., 2017	Peach aphid (*Myzus persicae*)	Insecta	Spotted lady beetle (*Coleomegilla maculata*)	Morphology	Real predator	Mother	2	Adult	Adult
Sentis et al., 2018	Pea aphid (*Acyrthosiphon pisum*)	Insecta	Asian lady bettle (*Harmonia axyridis*)	Morphology	Real predator	Mother	5	Entire life	Adult
Sentis et al., 2019	Pea aphid (*Acyrthosiphon pisum*)	Insecta	Asian lady bettle (*Harmonia axyridis*)	Morphology	Real predator	Mother	2	Adult	Larval/juvenile Adult
Sheriff et al., 2009	Snowshoe hare (*Lepus americanus*)	Mammalia	Dog (*Canis lupus*)	Life-history	Real predator	Mother	2	Adult	Neonates Larval/juvenile
Sheriff et al., 2010	Snowshoe hare (*Lepus americanus*)	Mammalia	Lynx (*Lynx canadensis*)	Physiology	Real predator	Mother	2	Entire life	Larval/juvenile
Shine and Downes, 1999	Scincid lizard (*Pseudemoia pagenstecheri*)	Squamata	White-lipped snake (*Drysdalia coronoides*)	Behavior Life-history Morphology	Chemical	Mother	2	Adult	Larval/juvenile

(Continued)

TABLE 1 | Continued

References	Prey species	Taxonomy	Predator species	Offspring traits	Cues	Parental sex exposed	Number of generations concerned	Parental stage exposed	Offspring stage concerned
St-Cyr and McGowan, 2015	Mice (*Mus musculus*)	Mammalia	Bobcat (*Lynx rufus*) Coyote (*Canis latrans*) Fox odor TMT (2,3,5-Trimethyl-3-thiazoline)	Behavior Life-history Morphology Genomic Physiology	Chemical	Mother	2	Adult	Larval/juvenile Adult
Stein and Bell, 2014	Three-spined stickleback (*Gasterosteus aculeatus*)	Actinopterygii	Northern pike (*Esox lucius*)	Behavior Life-history Morphology Physiology	Visual	Father	2	Adult	Adult
Stein et al., 2018	Three-spined stickleback (*Gasterosteus aculeatus*)	Actinopterygii	Northern pike (*Esox lucius*)	Behavior Genomic Life-history Morphology	Visual	Father	2	Adult	Larval/juvenile
Storm and Lima, 2010	Field cricket (*Gryllus pennsylvanicus*)	Insecta	Wolf spider (*Hogna helluo*)	Behavior Life-history	Real predator	Mother	2	Adult	Larval/juvenile
Stratmann and Taborsky, 2014	Mouthbrooding cichlid (*Simochromis pleurospilus*)	Actinopterygii	African cichlid (*Ctenochromis horei*)	Behavior Life-history Morphology Physiology	Real predator	Mother	2	Adult	Larval/juvenile
Tariel et al., 2019	Freshwater snail (*Physa acuta*)	Gastropoda	Spinycheek crayfish (*Orconectes limosus*)	Behavior	Chemical	Biparental	2	Larval/juvenile Adult	Adult
Tariel et al., 2020	Freshwater snail (*Physa acuta*)	Gastropoda	Spinycheek crayfish (*Orconectes limosus*)	Behavior Morphology	Chemical	Biparental	3	Larval/juvenile Adult	Adult
Tollrian, 1995	Water flea (*Daphnia pulex*)	Branchiopoda	Phantom midge (*Chaoborus punctipennis*)	Life-history	Chemical	Mother	2	Entire life	Larval/juvenile
Walsh et al., 2015	Water flea (*Daphnia ambigua*)	Branchiopoda	Redbreast sunfish (*Lepomis auritus*)	Life-history	Chemical	Mother	4	Entire life	Adult
Walsh et al., 2016	Water flea (*Daphnia ambigua*)	Branchiopoda	Anadromous alewive (*Alsoa pseudoharengus*)	Life-history	Chemical	Biparental	2	Entire life	Adult
Weisser et al., 1999	Pea aphid (*Acyrthosiphon pisum*)	Insecta	Seven-spot lady bettle (*Coccinella septempunctata*) Two-spot lady bettle (*Adalia bipunctata*)	Morphology	Real predator	Mother	2	Adult	Adult
Yin et al., 2015	Rotifer (*Brachionus calyciflorus*)	Rotifera	Freshwater rotifer (*Asplanchna brightwellii*)	Morphology	Chemical	Mother	3	Embryonic	Adult

TABLE 2 | Number of traits investigated across the 55 studies reviewed and putative adaptive value of the predator-induced transgenerational effects according to the trait type.

Trait type	Number of traits(anti-predator defenses)	Adaptive	Maladaptive	Unknown	No effect	Not relevant
Behavior	41 (35)	22	7	1	11	0
Morphology	42 (21)	20	2	8	12	0
Life-history	49 (11)	12	6	8	19	4
Physiology	10 (0)	2	0	5	3	0
Genomic	5 (0)	0	0	4	1	0
Epigenomic	3 (0)	0	0	3	0	0
Total (anti-predator defenses)	150 (67)	56 (41)	15 (9)	29 (2)	46 (12)	4 (3)

The numbers in brackets are traits involved in anti-predator defenses. The putative adaptive value (Adaptive, Maladaptive, Unknown) was defined according to the author's explanations although most studies did not directly assess the fitness consequences of predator-induced TGP. 'Not relevant' means that it makes no sense to characterize the adaptive value of a trait (e.g., survival). We do not consider the traits that referred to reproductive performances of parents (e.g., egg size or clutch size).

patterns during embryonic development (carry-over effect), or that increased timidity (a transgenerationally induced anti-predator behavior) involved a trade-off with foraging, limiting feeding of offspring from predator-exposed fathers. In these cases, we followed the study authors in classifying the traits as anti-predator defenses (i.e., participating in anti-predator strategy) or not (**Table 3**). The empirical studies included in this review reported statistically significant predator-induced TGP for 55 of the 68 (81%) anti-predator defenses and for 50 of the 82 (61%) more general responses (**Tables 2**, **3**), although this rather high prevalence may be influenced by publication bias.

Induction of anti-predator defenses both within and across generations depends mainly on the cost/benefit balance in different environments (with and without predators) (Uller, 2008; Auld et al., 2010; Murren et al., 2015). Benefits of induced responses can rely on (1) a decrease in the predator's ability to perceive the prey (behavioral traits: e.g., increased refuge use, decreased activity); for example, Freinschlag and Schausberger (2016) showed in the two-spotted spider mite (*Tetranychus urticae*) that maternal exposure to predator-cues changes offspring preference for feeding sites from the leaf vein (predator-free mothers) to the leaf blade (predator-exposed mothers), reducing the likelihood of encountering predators; (2) a deviation from the predator's preferred prey size (morphological and life-history traits: e.g., refuge-size, acceleration in development time, growth rate; Sogard, 1997); for example, Stratmann and Taborsky (2014) showed in a mouthbrooding cichlid (*Simochromis pleurospilus*) that offspring from predator-exposed mothers grow faster, protecting them from gape-limited fish predators; (3) an increased escape ability (morphological traits: e.g., improving locomotion ability or increasing handling time of predators to increase escape probability); for example, Bestion et al. (2014) exposed gravid females of common lizards (*Zootoca vivipara*) to snake cues and observed that offspring from predator-exposed mothers had longer tails (luring effect). Costs associated with induction of defenses within a generation arise mainly from changes in energy allocation. To our knowledge, costs of transgenerationally induced defenses have never been investigated, but are most likely similar to those of WGP. In addition, an interesting open question is whether the fact of transmitting defenses to offspring incurs costs to the parents.

Induction of anti-predator defenses also depends on trait lability, i.e., how fast the trait can change (induction and reversion) relative to predation risk; for example, behavioral traits are more labile and more easily reversible than morphological traits, which are in most cases more constrained during development and exhibit irreversible variations (West-Eberhard, 2003; Ghalambor et al., 2010). Theory therefore predicts that TGP should be found more frequently for morphological traits (allowing offspring to orient their early development toward an anti-predator response based on parental cues, before the morphological traits are fixed) than in behavioral traits (which should be more likely to be influenced by current cues) (Piersma and Drent, 2003; Dingemanse and Wolf, 2013; Kuijper and Hoyle, 2015). However, contrary to this theoretical prediction, the empirical studies included in this review found significant predator-induced TGP for all kinds of anti-predator defenses, including behavior [18 out of the 21 (86%) morphological defenses, 25 out of the 35 (71%) behavioral defenses and 10 out of the 11 (91%) life-history defenses, **Tables 2**, **3**]. The benefit of inducing anti-predator behavior across generations may be that, precisely because they are highly labile, they can be quickly "switched off" (due to WGP) in case of a mismatch between parental and offspring environments (Beaman et al., 2016). Conversely, TGP involving morphological defensive traits or life-history traits is likely to irreversibly engage the offspring in certain developmental trajectories, even if predation risk decreases.

Overall, our review shows that the effects of parental exposure to predation are not restricted to specific types of traits. However, within studies, the transgenerational effects vary strongly among traits (significance and direction: e.g., Luquet and Tariel, 2016; Donelan and Trussell, 2018a; Stein et al., 2018), meaning that interpretations based on single traits will often be meaningless. Instead, predator-induced TGP should be interpreted in the context of a global anti-predator strategy, including several types of traits, and if relevant, their anti-predator role, and how they trade off with each other.

EVOLUTIONARY ASPECTS OF PREDATOR-INDUCED TRANSGENERATIONAL PLASTICITY

A key question for the study of TGP in predator-prey interactions is whether the transgenerationally induced phenotype actually increases offspring fitness. Indeed, the evolutionary potential of

TABLE 3 | Nature and details of all traits involved in predator-induced TGP (anti-predator defenses themselves or not) in the 55 studies.

References	Trait nature	Anti-predator defense	Precise traits	Direction of TGP response	Trait-specific adaptive value	Global adaptive value	Interplay WGP and TGP	Direction WGP and TGP	TGP dependent on offspring environment
Agrawal et al., 1999	Morphology	Yes	Relative helmet length	Positive	Adaptive	Adaptive	Additive	Same direction	/
Basso et al., 2014	Life-history	No	Mass	No effect	No effect	Unknown			
	Life-history	No	Fledging success	Not relevant	Unknown				
	Life-history	No	Fledging age	No effect	No effect				
	Morphology	No	Tarsus length	Not relevant	Unknown				
	Morphology	No	Wing length	No effect	No effect				
Basso and Richner, 2015a	Life-history	No	Mass at hatching	Negative	Maladaptive	No effect			
	Life-history	No	Fledging mass	No effect	No effect				
	Life-history	No	Fledging success	No effect	No effect				
	Life-history	No	Fledging age	No effect	No effect				
	Morphology	No	Tarsus length	No effect	No effect				
	Morphology	No	Wing length	No effect	No effect				
Basso and Richner, 2015b	Life-history	No	Growth	Negative	Maladaptive	Maladaptive			
	Life-history	No	Fledging success	No effect	No effect				
	Life-history	No	Fledging age	No effect	No effect				
	Morphology	No	Tarsus growth	No effect	No effect				
Beaty et al., 2016	Behavior	Yes	Escape behavior	No effect	No effect	Adaptive	No TGP	No TGP	/
	Morphology	Yes	Crush-resistance	Positive	Adaptive		No WGP	No WGP	/
	Morphology	Yes	Shell shape	No effect	No effect		No TGP	No TGP	/
	Morphology	No	Shell size	Positive	Adaptive		Additive	Opposite direction	/
Bestion et al., 2014	Behavior	Yes	Activity	Positive	Maladaptive	Adaptive			
	Behavior	Yes	Dispersal	Positive	Adaptive				
	Behavior	No	Preferred temperature	Not relevant	Adaptive				
	Morphology	Yes	Tail length	Positive	Adaptive				
	Morphology	No	Total length	No effect	No effect				
	Life-history	No	Mass	No effect	No effect				
Coslovsky and Richner, 2011	Behavior	Yes	Dispersal	No effect	No effect	Adaptive			
	Life-history	No	Mass	Negative	Adaptive				
	Morphology	No	Sternum growth	No effect	No effect				
	Morphology	No	Tarsus length	Negative	Adaptive				
	Morphology	No	Wing length	Positive	Adaptive				
Coslovsky and Richner, 2012	Life-history	No	Growth	Unknown	Unknown	Unknown	Non-additive	Unknown	Unknown
	Life-history	No	Fledging success	No effect	No effect		No TGP	No TGP	/
	Life-history	No	Fledging age	Unknown	Unknown		Non-additive	Unknown	Unknown
	Morphology	No	Tarsus growth	Unknown	Unknown		Non-additive	Unknown	Unknown
	Morphology	No	Wing growth	Unknown	Unknown		Non-additive	Unknown	Unknown
Dixon and Agarwala, 1999	Morphology	Yes	Proportion of winged adult (red and green pea aphids)	Positive	Adaptive	Adaptive			

(Continued)

TABLE 3 | Continued

References	Trait nature	Anti-predator defense	Precise traits	Direction of TGP response	Trait-specific adaptive value	Global adaptive value	Interplay WGP and TGP	Direction WGP and TGP	TGP dependent on offspring environment
Donelan and Trussell, 2015	Behavior	Yes	Refuge use	Negative	Maladaptive	Adaptive	Non-additive	Opposite direction	Only in predator-cue
	Behavior	No	Foraging	Positive	Adaptive		Non-additive	Opposite direction	Only in predator-cue
	Physiology	No	Tissue maintenance	Positive	Adaptive		Non-additive	Opposite direction	Only in predator-cue
Donelan and Trussell, 2018a	Behavior	Yes	Refuge use	Negative	Maladaptive	Adaptive	No WGP	No WGP	/
	Behavior	No	Foraging	No effect	No effect		No WGP and No TGP	No WGP and No TGP	/
	Life-history	No	Growth efficiency	Positive	Adaptive		Additive	Direction WGP unknown	/
	Physiology	No	Tissue growth	Positive	Adaptive		Non-additive	Same direction	Only in predator-cue
Donelan and Trussell, 2018b	Life-history	No	Size at hatching	Positive	Adaptive	Adaptive	Non-additive	Direction WGP depends on TGP	Only in predator-cue
Dzialowski et al., 2003	Morphology	Yes	Head and tail spines	Positive	Adaptive	Adaptive			
Evans et al., 2007	Behavior	Yes	Capture with a hand net	No effect	No effect	Maladaptive			
	Behavior	Yes	Response time	No effect	No effect				
	Behavior	Yes	Schooling	No effect	No effect				
	Behavior	Yes	Swimming speed	Negative	Maladaptive				
	Morphology	No	Length	No effect	No effect				
Freinschlag and Schausberger, 2016	Behavior	Yes	Activity	No effect	No effect	Adaptive	No TGP	No TGP	/
	Behavior	Yes	Feeding site choice	Not relevant	Adaptive		No WGP	No WGP	/
	Life-history	No	Developmental time	Positive	Maladaptive		Non-additive	Direction WGP depends on TGP	Higher in control
Giesing et al., 2011	Behavior	Yes	Shoaling behavior	Positive	Adaptive	Adaptive			
	Life-history	No	Growth	No effect	No effect				
	Physiology	No	Egg metabolic rate	Positive	Unknown				
Goeppner et al., 2020	Morphology	Yes	Crush resistance	Negative	Maladaptive	Maladaptive			
	Morphology	Yes	Shell shape	Negative	Maladaptive				
Hales et al., 2017	Genomic	No	Gene expression	Not relevant	Unknown	Unknown			
Hellmann et al., 2019	Behavior	Yes	Activity (sons; paternal treatment)	Positive	Maladaptive	Unknown			
	Behavior	No	Scototaxis (maternal treatment)	Positive	Adaptive				
	Genomic	No	Brain gene expression	Not relevant	Unknown				
	Life-history	Yes	Captures by predator (paternal treatment)	Positive	Not relevant				
Hellmann et al., 2020	Behavior	Yes	Activity (daughers; paternal grandfather treatment)	Negative	Adaptive	Unknown			
	Behavior	Yes	Escape behavior (sons; maternal grandfather treatment)	Negative	Maladaptive				
	Behavior	Yes	Freezing (sons; maternal grandfather treatment)	Negative	Maladaptive				
	Life-history	No	Mass (size-corrected; daughters)	Positive	Adaptive				

(Continued)

TABLE 3 | Continued

References	Trait nature	Anti-predator defense	Precise traits	Direction of TGP response	Trait-specific adaptive value	Global adaptive value	Interplay WGP and TGP	Direction WGP and TGP	TGP dependent on offspring environment
	Physiology	No	Plasma cortisol	No effect	No effect				
Keiser and Mondor, 2013	Behavior	Yes	Feeding site choice	Positive	Adaptive	Adaptive			
Kunert and Weisser, 2003	Morphology	Yes	Proportion of winged adult	Positive	Adaptive	Adaptive			
Kunert and Weisser, 2005	Morphology	Yes	Proportion of winged adult	Positive	Adaptive	Adaptive			
Lehto and Tinghitella, 2019	Life-history	No	Daughter's egg size	No effect	No effect	Unknown			
	Life-history	No	Daughter's egg number	No effect	No effect				
	Physiology	No	Daughter's egg cortisol	Positive	Unknown				
Li and Zhang, 2019	Life-history	No	Hatching age	No effect	No effect	Unknown			
	Life-history	No	Development	No effect	No effect				
	Life-history	No	Survival	Negative	Not relevant				
	Life-history	No	Lifespan	No effect	No effect				
	Life-history	No	Reproduction	No effect	No effect				
Luquet and Tariel, 2016	Behavior	Yes	Escape behavior	Positive	Adaptive	Adaptive	Non-additive	Same direction	Only in control
	Morphology	Yes	Shell shape	No effect	No effect		Non-additive	Direction WGP depends on TGP	/
	Morphology	Yes	Shell thickness	Positive	Adaptive		Non-additive	Same direction	Only in control
	Morphology	No	Shell size	Negative	Unknown		Additive	Same direction	/
	Morphology	No	Mass	Negative	Unknown		Additive	Same direction	/
McGhee and Bell, 2014	Behavior	No	Anxiety	Not relevant	Unknown	Unknown			
	Epigenomic	No	Epigenetic alteration	Not relevant	Unknown				
McGhee et al., 2012	Behavior	Yes	Avoidance behavior	Negative	Adaptive	Adaptive			
	Life-history	Yes	Survival	Negative	Not relevant				
Mikulski and Pijanowska, 2010	Life-history	Yes	Clutch size	Negative	Adaptive	Adaptive	Non-additive	Direction TGP unknown	Both in control and predator-cue
	Life-history	Yes	Size at maturation	Negative	Adaptive		Non-additive	Direction TGP unknown	Both in control and predator-cue
Mommer and Bell, 2013	Physiology	No	Plasma cortisol	Not relevant	Unknown	Unknown			
Mondor et al., 2005	Morphology	Yes	Proportion of winged adult	Positive	Adaptive	Adaptive			
Monteforte et al., 2020	Genomic	No	Telomere length	No effect	No effect	Unknown			
	Life-history	No	Size at birth	Negative	Unknown				
Morales et al., 2018	Behavior	Yes	Freezing (second-laid eggs)	Positive	Adaptive	Adaptive			
	Behavior	Yes	Response time (second-laid eggs)	Negative	Adaptive				
	Life-history	No	Mass at hatching (third-laid eggs)	Negative	Maladaptive				
Podjasek et al., 2005	Morphology	Yes	Proportion of winged adult	Positive	Adaptive	Adaptive			
Schield et al., 2016	Epigenomic	No	Methylation patterns	Not relevant	Unknown	Unknown			
Segers et al., 2011	Life-history	No	Size	No effect	No effect	No effect			
Seiter and Schausberger, 2015	Behavior	Yes	Activity	Negative	Adaptive	Adaptive			
	Behavior	Yes	Feeding site choice	Positive	Adaptive				

(Continued)

TABLE 3 | Continued

References	Trait nature	Anti-predator defense	Precise traits	Direction of TGP response	Trait-specific adaptive value	Global adaptive value	Interplay WGP and TGP	Direction WGP and TGP	TGP dependent on offspring environment
Sentis et al., 2017	Morphology	Yes	Proportion of winged adult	Positive	Adaptive	Adaptive			
Sentis et al., 2018	Morphology	Yes	Proportion of winged adult	Positive	Adaptive	Adaptive			
Sentis et al., 2019	Morphology	Yes	Proportion of winged adult	Positive	Adaptive	Adaptive			
Sheriff et al., 2009	Life-history	No	Right hind foot (RHF) length	Negative	Maladaptive	Maladaptive			
	Life-history	No	Mass	Negative	Maladaptive				
Sheriff et al., 2010	Physiology	No	Plasma cortisol and maximum corticosteroid-binding capacity	Positive	Unknown	Unknown			
Shine and Downes, 1999	Behavior	Yes	Tongue-flick	Positive	Adaptive	Adaptive			
	Behavior	No	Speed	No effect	No effect				
	Life-history	No	Mass	Positive	Unknown				
	Morphology	Yes	Tail length	Positive	Adaptive				
St-Cyr and McGowan, 2015	Behavior	Yes	Avoidance behavior	Positive	Adaptive	Adaptive			
	Behavior	Yes	Activity	Negative	Adaptive				
	Genomic	No	Brain stress-related gene expression	Not relevant	Unknown				
	Epigenomic	No	Brain DNA methylation	Not relevant	Unknown				
	Life-history	No	Mass	No effect	No effect				
	Morphology	No	Size	No effect	No effect				
	Physiology	No	Cortisol	Positive	Unknown				
Stein and Bell, 2014	Behavior	Yes	Activity	Negative	Adaptive	Unknown			
	Behavior	Yes	Freezing	No effect	No effect				
	Behavior	Yes	Jerky swimming	No effect	No effect				
	Life-history	No	Mass	Negative	Unknown				
	Morphology	No	Size	Negative	Unknown				
	Morphology	No	Color (size-corrected)	No effect	No effect				
	Physiology	No	Plasma cortisol	No effect	No effect				
Stein et al., 2018	Behavior	Yes	Response time	Positive	Adaptive	Adaptive	Non-additive	Same direction	Only in control
	Genomic	No	Brain gene expression	Not relevant	Unknown		Non-additive	Same direction	Only in control
	Life-history	No	Mass	Negative	Unknown		Non-additive	Same direction	Only in control
	Morphology	No	Size	Negative	Unknown		Non-additive	Same direction	Only in control
Storm and Lima, 2010	Behavior	Yes	Freezing	Positive	Adaptive	Adaptive			
	Life-history	Yes	Survival	Positive	Not relevant				
Stratmann and Taborsky, 2014	Behavior	Yes	Response time, freezing, avoidance and escape behavior	No effect	No effect	Adaptive	No WGP and No TGP	/	/
	Life-history	No	Growth	Positive	Adaptive		No WGP	/	/
	Morphology	No	Size	Positive	Adaptive		No WGP	/	/
	Physiology	No	Opercular beat rate	No effect	No effect		No TGP	/	/

(Continued)

TABLE 3 | Continued

References	Trait nature	Anti-predator defense	Precise traits	Direction of TGP response	Trait-specific adaptive value	Global adaptive value	Interplay WGP and TGP	Direction WGP and TGP	TGP dependent on offspring environment
Tariel et al., 2019	Behavior	Yes	Escape behavior	Negative	Adaptive	Adaptive	Additive	Same direction	/
Tariel et al., 2020	Behavior	Yes	Escape behavior	Positive	Adaptive	Unknown	Additive	Same direction	Both in control and predator-cue
	Morphology	Yes	Shell thickness	Not relevant	Unknown		Non-additive	Direction TGP depends on WGP	Both in control and predator-cue
	Morphology	No	Shell size	No effect	No effect		No TGP	No TGP	/
Tollrian, 1995	Life-history	Yes	Size at birth	Positive	Unknown	Adaptive			
	Life-history	No	Size at maturation	Positive	Adaptive				
Walsh et al., 2015	Life-history	Yes	Age at maturation	Negative	Adaptive	Adaptive			
	Life-history	Yes	Clutch size	Positive	Adaptive				
Walsh et al., 2016	Life-history	Yes	Age at maturation	Negative	Adaptive	Adaptive			
	Life-history	Yes	Clutch size	No effect	No effect				
	Life-history	Yes	Size at maturation	Negative	Adaptive				
Weisser et al., 1999	Morphology	Yes	Proportion of winged adult	Positive	Adaptive	Adaptive			
Yin et al., 2015	Morphology	Yes	Length of posterolateral spine	Positive	Adaptive	Adaptive	Additive	Same direction	/

The direction of TGP responses (e.g., positive: increase in the trait value with parental exposure to predator-cues) and the global and trait-specific adaptive values are indicated. For 13 studies, where both WGP and TGP were investigated in offspring, we specified the type (additive, non-additive effects) and direction of the TGP x WGP interplay.

TGP depends on its adaptive value, the correlation between parental and offspring predation risk and on how the cue of predation risk is imprinted at a molecular level and inherited over generations.

Adaptive Value

Determining the adaptive value of TGP is not an easy task, because depending on studies, parental exposure to predation can have very different outcomes with regard to offspring fitness. In some cases, transgenerational responses appear clearly adaptive, for example, when offspring of predator-exposed parents exhibit stronger inducible defenses (e.g., Agrawal et al., 1999; Storm and Lima, 2010; Luquet and Tariel, 2016; **Table 3** – "Adaptive value" columns). In other cases, transgenerational responses are more complex and depend on interactions between ancestral and offspring environments, the trait under consideration (§2; Tariel et al., 2020), developmental stage of offspring (§4.1.1; Li and Zhang, 2019) and sex (§5.1; Hellmann et al., 2019), and are therefore more difficult to relate to self-explanatory anti-predator scenarios with clear adaptive advantages. In addition, detection of predator presence may generate stress, which not only negatively impacts parental condition, but may also generate negative carry-over effects on offspring (state-based TGP, e.g., reduced body condition of progeny; Stein et al., 2018). As only three studies measured offspring fitness experimentally (for instance, with a survival test against a lethal exposure to predators; Storm and Lima, 2010; McGhee et al., 2012; Hellmann et al., 2019), we determined putative adaptive values of the transgenerational responses to predation by following the opinion of the study authors (**Tables 2**, **3**). 64% of studies reported potentially adaptive responses, while 7% reported maladaptive responses; for 25% of studies, we were not able to determine the adaptive nature of the induced trait(s), and two studies did not show any transgenerational effects at all (**Table 3** – "Global adaptive value" column). Although this imbalance may be due to publication bias in favor of systems with well-characterized anti-predator defenses, the above pattern nevertheless shows that predator-induced TGP has the potential to play an important role in predator-prey interactions, by allowing prey to pre-adapt their phenotype to future predation risk. Concerning interactions between ancestral and offspring environments, 14 studies report such interactions using full factorial experiments (**Table 3** – "Interplay WGP and TGP" column). Results were very varied: WGP and TGP can operate in the same or opposite directions (**Table 3** – "Direction WGP and TGP" column). Moreover, the direction of WGP can depend on parental environments, and conversely, the direction of TGP can depend on offspring environments (**Table 3** – "Direction WGP and TGP" column). Interestingly, WGP can mask TGP, i.e., the effects of parental environment are only observed in predator-free offspring environments, or reveal it, i.e., the effects of parental environment are only observed in offspring experiencing predator presence (**Table 3** – "TGP dependent on offspring environment" column), while TGP can override WGP, i.e., offspring do not respond to their own environment

(e.g., Beaty et al., 2016; Freinschlag and Schausberger, 2016; Luquet and Tariel, 2016).

Evolutionary Implications

The next question is then to know whether predator-induced TGP can evolve and under what environmental conditions. In general, phenotypic plasticity is selected when the environment is variable in time and/or space and provides reliable and accurate cues about future selection pressures that will act on the induced phenotype (Kuijper and Hoyle, 2015; Leimar and McNamara, 2015). This is challenging for TGP, because cue perception and expression of the induced phenotype are distant in time with a minimum lag time of one generation. Empirical examples showing that TGP can evolve are rare, but some examples come indeed from the context of predator-induced defenses. Two studies have demonstrated local adaptation of transgenerational responses to predators in wild populations. Storm and Lima (2010) showed that gravid crickets (*Gryllus pennsylvanicus*) from populations with predators produce offspring that are more responsive to predator-cues than those from populations without predators. Walsh et al. (2016) studied induced defenses of *Daphnia ambigua* in populations under three regimes of fish predation (consistently strong, consistently weak, or variable predation risk). They demonstrated that consistently strong (or weak) predation risk selected for TGP, while variable risk favored WGP. These two examples confirm that TGP may evolve in the wild, and that temporal variability and predictability of predation risk are key forces driving evolution of predator-induced TGP. In contrast, Goeppner et al. (2020) found *Physa acuta* snails from a population with predators to be more crush-resistant than snails from a population without predators (local adaptation), but no differential patterns of predator-induced TGP between the two populations (same transgenerational responses in both populations, and opposite to WGP). Finally, evolution of predator-induced TGP is suggested by two studies on aphids (*Acyrthosiphon pisum*). Sentis et al. (2019) compared clonal lineages specialized on two host plants characterized by contrasting predation risk. They found that lineages specialized on plants associated with high predation risk had a stronger transgenerational response to predators (increased frequency of winged offspring) relative to those from plants associated with low predation risk. Importantly, the authors showed that the proportion of winged offspring has high heritability, indicating that this defense and its plasticity can potentially evolve by selection. In another study, Sentis et al. (2018) exposed aphids from the same clone to predator presence or absence for 27 generations. They observed that predator-induced TGP was similar between treatments after 16 generations of predator exposure, but decreased after 25 generations of predator exposure in the predator treatment compared to the predator-free treatment, suggesting that TGP was counter-selected (probably because production costs of defenses were not compensated by higher survival to predators in their experimental system where dispersal was limited). With so few studies, it seems difficult to draw general conclusions about the evolutionary potential of predator-induced TGP, but both theoretical and empirical studies point in the same direction. Therefore, it is crucial to investigate the evolutionary potential of predator-induced TGP by (1) assessing the heritability of transgenerational responses to predators, (2) demonstrating in the laboratory (e.g., by experimental evolution) that TGP can be selected according to the variability and predictability of predation risk, and (3) confirming local adaptation in transgenerational responses to predators in wild populations.

Inheritance Mechanisms

While it is now well-established that predation can induce defensive responses that persist for several generations, the mechanisms underlying this persistence remain a black box. For example, the relationship between shifts in gene-expression patterns and defense induction has only been described within a generation (Miyakawa et al., 2010; Tollrian and Leese, 2010). For transmission of information across generations, many authors evoke non-genetic sources of heritability, especially epigenetic ones (Boškoviæ and Rando, 2018; Norouzitallab et al., 2019; Duempelmann et al., 2020). However, both genetic and epigenetic mechanisms of predator-induced TGP have rarely been investigated in detail. Indeed, we found only six studies that explored predator-induced TGP at the genomic level (McGhee and Bell, 2014; St-Cyr and McGowan, 2015; Schield et al., 2016; Hales et al., 2017; Stein et al., 2018; Hellmann et al., 2019). Hales et al. (2017) tested the influence of chemical cues from fish predators on gene expression patterns within and across three generations (F0, F1, and F2) in *D. ambigua*. The clone they used was known to show strong predator-induced TGP, with phenotypic changes opposite to those induced by WGP (Walsh et al., 2015). They found that TGP and WGP involved changes in expression level in different sets of genes, indicating divergent underlying mechanisms. In contrast, Stein et al. (2018) showed that TGP and WGP involved changes in identical sets of genes in offspring of three-spined stickleback (*Gasterosteus aculeatus*), and that changes in expression level of these genes were identical whether predation risk was experienced by the father (TGP), the offspring (WGP) or both. At the phenotypic level, WGP and TGP also involved identical responses. These two studies suggest that predation risk induces similar genomic responses within and across generations when the phenotypic responses are themselves similar. In addition, genomic mechanisms may differ between the sexes. Hellmann et al. (2019) demonstrated in *G. aculeatus* that maternal and paternal exposure to predation risk had distinct effects on gene expression patterns in the offspring brain, and that these effects varied between male and female offspring. A similar result was observed in mice, where only the gene expression patterns of female offspring were impacted by maternal exposure to predator-cues (St-Cyr and McGowan, 2015).

Interestingly, Hales et al. (2017) observed a decrease in the number of differentially expressed genes between the F1 and F2 generations—a trend consistent with the observed decrease in transgenerational responses (§4.2; Walsh et al., 2015) and the lability of inherited epigenetic marks (Fallet et al., 2020). In a companion methodological paper, Schield et al. (2016) found shifts in the methylation state of sampled loci between F0 (with predator-cues) and F1 (without predator-cues) in *D. ambigua*, suggesting that DNA methylation patterns

can vary between generations experiencing different predation environments. However, their experimental design dit not allow to demonstrate whether the epigenetic modifications are (1) sensitive to predator-cues (with vs. without predator-cues within a generation) and (2) related to the transmission of predation risk across generations (this would require a fully factorial design across two generations). In addition, Hellmann et al. (2020) showed that F0 exposure to predator-cues influenced the phenotype of F2 but not F1, indicating that epigenetic transmission and phenotypic consequences can be decoupled. Individuals could be carriers of epigenetic information and transmit altered phenotypes to their offspring without displaying the phenotypes themselves. Finally, McGhee and Bell (2014) showed in *G. aculeatus* that the amount of direct care provided by fathers, when modulated by predation risk, was linked to differential expression in offspring brains of a DNA methyltransferase (Dnmt3a) responsible for *de novo* methylation. Although this is not evidence of transgenerational epigenetic inheritance, it suggests that predation-risk driven behavior of fathers may influence the epigenetic programming of their offspring, which might in turn be transmitted to the next generation. To our knowledge, how and to what extent the epigenome is related to phenotype across generations is still an open question both in predator-prey systems and in general (see Fallet et al., 2020 for a detailed discussion). Taken together, the above results highlight the need for future work examining predator-induced TGP and WGP simultaneously, at both the (epi)genomic and phenotypic levels.

TEMPORAL DYNAMICS OF PREDATOR-INDUCED TRANSGENERATIONAL PLASTICITY

Transgenerational plasticity is a temporal process that is initiated in past generations, but has consequences in current and future generations. Temporal dynamics are thus a key aspect of this process: what are the time windows during which an environmental signal must be detected to be transmitted across generations? When are the transgenerational responses expressed in offspring? How long can predator-induced TGP persist in prey across generations?

Critical Time Windows to Trigger Transgenerational Plasticity

Evolution of TGP depends on how accurately the parental cue eliciting TGP predicts the environment that will exert selection on the offspring phenotype (Auge et al., 2017; Bell and Hellmann, 2019; Donelan et al., 2020). Cue accuracy depends on the time lag between cue perception and expression of offspring phenotypes (§3.2). Therefore, two developmental aspects may influence the presence, strength and direction of predator-induced TGP: (1) the life-history stage at which parents perceive environmental cues (induction time), and (2) the life-history stage at which the offspring initiate responses (expression time) (**Figure 2**; Burton and Metcalfe, 2014; Donelson et al., 2018; Bell and Hellmann, 2019; Yin et al., 2019; Donelan et al., 2020). Among the 55 studies that explore transgenerational effects in response to predation risk, 18 investigate patterns of TGP with different timings of exposure in parents (four studies) or expression in offspring (14 studies).

Induction Time of Transgenerational Plasticity: Parental Sensitive Windows

The time at which an environmental change is experienced can directly influence phenotypic responses in both the exposed individual (WGP) and its offspring (TGP) (Burton and Metcalfe, 2014; Donelson et al., 2018; Donelan et al., 2020). Some theoretical models predict that early-perceived cues in parental development should have weaker effects on offspring than late-perceived cues (Ezard et al., 2014; Prizak et al., 2014). Indeed, later parental environments are better predictors of offspring environment because of the shorter time lag between perception of cues and the predicted environment. Therefore, the period before and during reproduction in parental development is expected to be a critical period for TGP induction (Donelan et al., 2020). In contrast, some empirical studies rather identify the early parental development (i.e., embryonic or neonate phases) as particularly sensitive to influence TGP (Burton and Metcalfe, 2014; Fawcett and Frankenhuis, 2015; Donelson et al., 2018; Yin et al., 2019), which might be linked to a higher proportion of early embryonic cells that are highly sensitive to environmental cues (Burton and Metcalfe, 2014).

In the context of predator-induced TGP, most studies (62%) focused on parental exposure during adult life only (**Table 1**). A few studies explored the effects of parental exposure during the post-embryonic period (neonates + larval/juvenile phase, larval/juvenile phase, larval/juvenile phase + adults; 2, 7, and 13% of studies, respectively) or TGP responses when parents were confronted with predator presence during their entire life including the embryonic phase (15%). Only one study was carried out with parents exposed to predator-cues only in the embryonic phase (Yin et al., 2015). Finally, we found only four studies (7%) that compared the influence of different exposure timings (Agrawal et al., 1999; Mikulski and Pijanowska, 2010; Walsh et al., 2015; Yin et al., 2015). Three of these studies are focused on water fleas (*D. cucullata, D. magna, D. ambigua*; Agrawal et al., 1999; Mikulski and Pijanowska, 2010; Walsh et al., 2015), while the fourth one is on the rotifer *Brachionus calyciflorus* (Yin et al., 2015). Considering these four studies, three patterns emerge (**Figure 2**).

Two studies demonstrated that the expression of predator-induced TGP depended on the parental life stage in which the cues were perceived, but timing differed between the two. Agrawal et al. (1999) showed that a defensive morphology in water fleas (*D. cucullata*) was induced in offspring from mothers exposed to predator-cues before becoming pregnant, but not in offspring from mothers exposed later. Walsh et al. (2015), working on *D. ambigua*, found that offspring from mothers exposed to predator-cues during the juvenile stage did not show TGP with respect to the age of maturation. In contrast, offspring from mothers that had been exposed during their entire life or

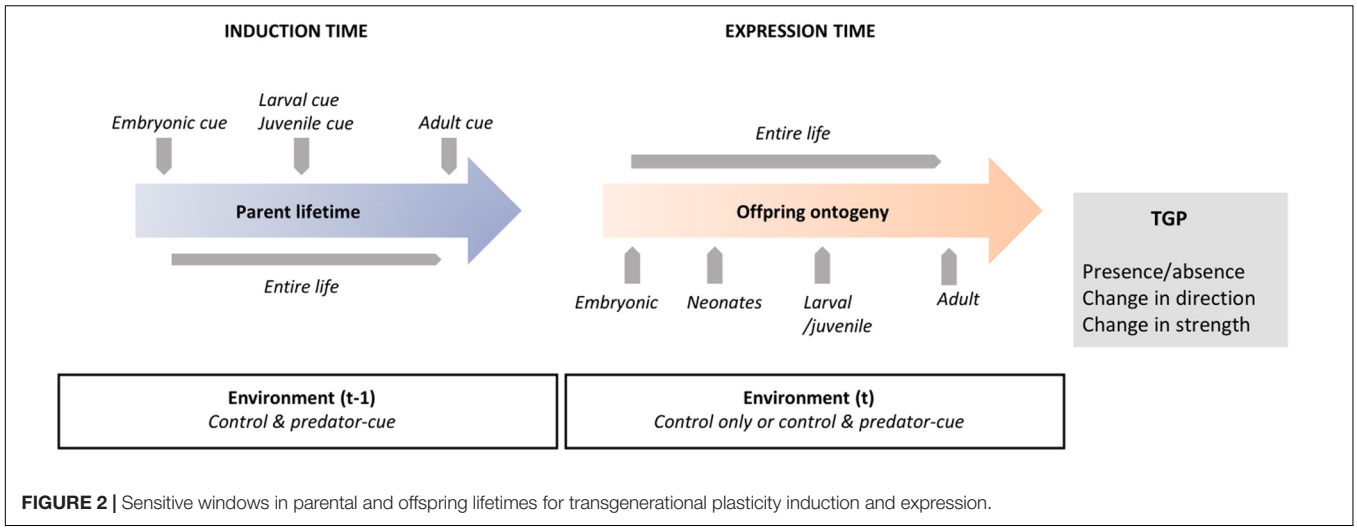

FIGURE 2 | Sensitive windows in parental and offspring lifetimes for transgenerational plasticity induction and expression.

only late in their development (i.e., at maturation) matured faster than those originated from predator-free mothers.

Two other studies highlighted that the strength of predator-induced TGP depended on the parental life stage in which the cues were perceived. Offspring from *D. magna* mothers exposed to predator-cues at a late juvenile stage exhibited broader life-history responses to predation (i.e., maturity at a smaller size, lower fecundity) than offspring from mothers confronted with predation at earlier or later life stages (Mikulski and Pijanowska, 2010). Similarly, offspring from *B. calyciflorus* mothers exposed to predator cues at a late embryonic stage exhibited broader morphological responses to predation (i.e., longer spine and higher posterolateral spine-body length ratio) during two generations than offspring from mothers exposed at earlier embryonic stages (Yin et al., 2015).

To our knowledge, no study has found evidence that the direction of predator-induced TGP depends on the parental life stage in which the cues are perceived. However, an element of a response emerges when two different studies on sticklebacks (*G. aculeatus*) are combined. While paternal exposure to predator-cues during sperm formation decreased offspring anti-predator behavior and survival in the presence of a real predator (Hellmann et al., 2019), paternal exposure later during egg care increased offspring anti-predator behavior and decreased body size and condition (Stein and Bell, 2014). These results suggest that, in the first case, fathers transmitted negative effects of predator-induced stress to their offspring, while in the second case, fathers pre-adapted their offspring to predation risk.

All these results confirm the importance of considering how the timing of parental exposure affects the presence, strength and direction of transgenerational phenotypic changes. Results are not consistent across studies and show that several parental life stages can be critical periods for influencing the next generation (embryonic stage, late juvenile stage, or at maturation), sometimes with a narrow window of sensitivity (early and late embryonic stage, or at maturation before or after being pregnant).

Expression Time of Transgenerational Plasticity: Offspring Sensitive Windows in Ontogeny

From the offspring's perspective, timing of cue perception is important because it determines when offspring can initiate an appropriate response to information transmitted by their parents. Some researchers expect a stronger effect on offspring phenotype when parental cues are received early in embryonic development (Bell and Hellmann, 2019). However, it is difficult to know when cues are actually perceived or integrated by the offspring, and studies generally only report the offspring stage at which TGP is expressed. The meta-analysis of Yin et al. (2019) found that TGP is most strongly expressed in offspring juvenile stages, and less strongly in embryonic and adult stages. In the context of predation, the majority of studies measured transgenerational responses only once during offspring development: during early development (neonates) in 11% of studies, later during the larval-juvenile stage in 29% of studies, and during the adult stage in 35% of studies (**Table 1**). No studies examined TGP expression only in the embryonic stage. To our knowledge, 14 studies (25%) explored TGP expression in response to predation risk at different times in offspring life, either within an offspring stage or among different stages (Tollrian, 1995; Agrawal et al., 1999; Sheriff et al., 2010; Coslovsky and Richner, 2011, 2012; Giesing et al., 2011; Basso et al., 2014; Bestion et al., 2014; Stratmann and Taborsky, 2014; Basso and Richner, 2015a,b; St-Cyr and McGowan, 2015; Freinschlag and Schausberger, 2016; Li and Zhang, 2019). Screening these studies, different scenarios are observed (**Figure 2**).

Five studies described that parental experience with predation risk can influence offspring traits early in development, but the effect dissipates later in life. Two studies on spider mites (*T. urticae*) showed that maternal predation experience retarded offspring development in embryonic, larval and early juvenile stages, but the effect disappeared in the late juvenile stage and for reproductive parameters in adults (Freinschlag and Schausberger, 2016; Li and Zhang, 2019). In the

same way, offspring of predator-exposed mothers were smaller and lighter during the early juvenile stage in great tits (Coslovsky and Richner, 2011, 2012; Basso and Richner, 2015a) and grew faster in an African cichlid (Stratmann and Taborsky, 2014), whereas no TGP of these traits was found later in the juvenile and adult stages for both models.

In the study of Agrawal et al. (1999), TGP was also expressed early in offspring development, but decreased later without disappearing completely. Indeed, differences in helmet length in *D. cucullata* offspring (second and third brood of the same generation) from mothers exposed to predator-cues relative to those from control mothers were stronger in the neonate stage than later in the adult stage. In contrast, Bestion et al. (2014) showed an increasing strength of TGP expression over offspring development: common lizard juveniles (*Z. vivipara*) born to mothers exposed to predator-cues had longer tails relative to body length already at birth, but the difference was stronger later in development. In the study of Basso and Richner (2015b) on great tits, maternal exposure to predator-cues did not significantly alter offspring growth rate at birth, but later during juvenile development.

Three other studies also showed that parental predator experience induces defenses in early life-history stages that persist over offspring development with approximately the same strength. For example, in Tollrian (1995), the difference in body size of *D. pulex* offspring from mothers exposed to predator-cues relative to offspring from control mothers remained significant and at approximately the same strength throughout juvenile development (six instars). In another species of water flea (*D. cucullata*), neonates (first brood) born from mothers exposed to predator-cues produced stronger induced defenses (i.e., higher relative helmet length) than neonates from mothers raised in a predator-free environment (Agrawal et al., 1999), and this effect persisted with the same strength when offspring reached maturity. In the same way, Sheriff et al. (2010) found identical patterns of TGP responses (increased fecal corticosteroid metabolite concentration, a stress index) for juvenile snowshoe hares (*L. americanus*) irrespective of their age.

In contrast, only one study (Coslovsky and Richner, 2011), on wing development in great tits (*P. major*), found transgenerational responses changing direction over the course of offspring development. The authors found that, just after birth, offspring from predator-exposed mothers had shorter wings than those from predator-free mothers. Later, however, wing growth in offspring from predator-exposed mothers was accelerated. Consequently, on day 14, the difference between the two treatments had vanished, and at age of maturity, offspring from predator-exposed mothers had longer wings than those from predator-free mothers.

Finally, to our knowledge, no studies to date found that parental experience of predator-cues can shape offspring phenotype strictly late in life. While many studies (42%) investigated and found TGP in adults, it is not possible to determine if these transgenerational effects appeared earlier and persisted across development or if they appeared only in the adult stage.

All these studies on expression time suggest that offspring can integrate past experiences of predation into the expression of their phenotype at different stages of development. Transgenerational effects in offspring may be expressed in a single stage or throughout the entire life, and their strength and direction can change during development. Contrary to the meta-analysis of Yin et al. (2019), which found that TGP tends to be weak in early offspring development and stronger later on during the juvenile stage, most studies reviewed here show that predator-induced TGP is stronger in early offspring stages.

In conclusion, empirical studies on the timing of TGP showed equivocal and often inconsistent results. Indeed, TGP may be induced by predator-cues perceived during different developmental windows in parents and may be expressed at different life stages in offspring. Missing these critical windows in experiments may lead to underestimation of the importance of TGP in predator-prey interactions.

Persistence Across Generations

How and for how long predator-induced TGP can persist in prey across generations is crucial information, because it determines the extent to which transgenerational responses contribute to long-term evolutionary changes. Since TGP is characterized by a lag time between cue perception and expression of the induced phenotype (§3.2), its persistence across generations should depend on the reliability of cues in predicting predation risk in subsequent generations. Based on the patterns proposed in the review of Bell and Hellmann (2019) on general TGP, we can propose three scenarios: (1) Cues are reliable indicators of predation risk only for the next generation: F1 offspring produced by parents exposed to predator-cues (F0 generation) express phenotypic changes, which dissipate in the F2 and subsequent generations (pattern 1 in **Figure 3**); (2) Cues are reliable indicators for several generations: the induced phenotype of the F1 generation persists in a similar way (strength and direction) across one (F2) or multiple generations (pattern 2 in **Figure 3**); (3) The reliability of cues decreases over time: the induced phenotype of the F1 generation persists across multiple generations, but with decreasing mean effects between consecutive generations (pattern 3 in **Figure 3**).

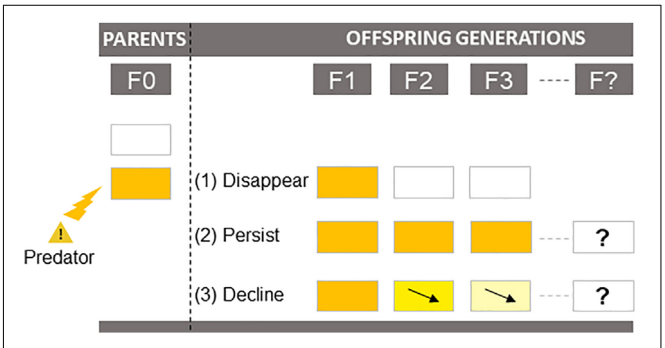

FIGURE 3 | Scenarios of transgenerational plasticity persistence over generations. Prey are exposed to predator cues only at the F0 generation.

Most of the studies reviewed here (87%) investigated predator-induced TGP over two generations only (F0 and F1). In the following, we summarize the results of seven studies that explored the influence of past exposure to predation over three (Agrawal et al., 1999; Yin et al., 2015; Hales et al., 2017; Hellmann et al., 2020; Tariel et al., 2020), four (Walsh et al., 2015) or five generations (Sentis et al., 2018). Agrawal et al. (1999) showed that the morphological defense of *D. cucullata* (relative helmet length) induced in F1 offspring from F0 predator-exposed parents disappeared in F2 offspring (pattern 1 in **Figure 3**). A similar pattern was found for clutch size in *D. ambigua* (Walsh et al., 2015), total length and stress-induced cortisol in the three-spined stickleback *G. aculeatus* (Hellmann et al., 2020), and for body mass and shell size in the freshwater snail *P. acuta* (Tariel et al., 2020): in all cases, the transgenerational effect disappeared in the F2 generation (no effect of the grand-parental environment). In contrast, Yin et al. (2015) demonstrated grand-maternal induction of defensive morphology (posterolateral spine) in rotifers *B. calyciflorus*. Similarly, Tariel et al. (2020) found that grand-parental exposure to predator-cues influenced escape behavior and shell thickness in *P. acuta*. For age at maturation in *D. ambigua*, predator-induced TGP (earlier maturation) was detectable two generations following cue removal (i.e., until the F2 generation), and finally disappeared in the F3 (Walsh et al., 2015). Interestingly, the transgenerational effect in the F2 generation was lower than in the F1 in all studies (Walsh et al., 2015; Yin et al., 2015; Tariel et al., 2020), indicating a decline of transgenerational response to predation over time (pattern 3 in **Figure 3**). Moreover, Hellmann et al. (2020) observed in *G. aculeatus* that predator-induced TGP persisted in a lineage-specific (through the grand-maternal or grand-paternal lineage) and in a sex-specific (only in male or female grand-offspring) way: F2 *females* were heavier and had a reduced anti-predator response (reduced activity after a simulated predator attack) when their *paternal* grandfather was exposed to predator-cues, while F2 *males* had a reduced anti-predator response (frozen and escape behavior) when their *maternal* grandfather was exposed to predator-cues. This means that transgenerational effects may selectively persist across generations in only a subset of individuals, which can make it very difficult to assess the persistence of predator-induced TGP, since most studies focused on average responses only. To our knowledge, the experimental-evolution experiment by Sentis et al. (2018) is the only study that investigated predator-induced TGP over five generations. They exposed genetically identical pea aphids (*A. pisum*) to predator presence for 27 generations, but removed predators at three points (after 3, 13, and 22 generations of exposure) and monitored predator-induced TGP for five generations after predator removal. They found that the defensive phenotype—a high frequency of winged aphids in the population—persisted for one generation after predator removal, but then fell for two to three generations below the level of the predator-free treatment (lower proportion of aphids with defenses). This example illustrates not only that TGP can persist over several generations, but also that the effects can change direction (potentially going from adaptive to non-adaptive) over generations. Interestingly, the number of generations needed to come back to the level of the predator-free treatment increased with the number of generations previously exposed to predators. This suggests that the accumulation of exposures to predators over generations may increase the persistence of predator-induced TGP.

Although there are too few studies to draw a general conclusion, all studies to date show that predator-induced TGP can extend beyond the generation following predator exposure, but seems to decline gradually with each generation and eventually disappear (pattern 3 on **Figure 3**). This highlights the need for empirical studies on longer timescales to determine how long the signals of predators are embedded across generations.

OTHER KEY ASPECTS OF PREDATOR-INDUCED TRANSGENERATIONAL PLASTICITY

According to the literature, two other aspects can influence the induction, strength and direction of predator-induced TGP: sex and the strength of predation risk. The key questions are: (1) Do paternal and maternal environments influence offspring phenotype in the same way or not? And do transgenerational responses depend on offspring sex? (2) Do prey invest in anti-predator responses according to the level of predation risk in the parental environment?

Sex-Specific Predator-Induced Transgenerational Plasticity

TGP can be sex-specific: its induction in offspring may depend on which parent transmits the environmental signal (parental level) and its expression by offspring may depend on their sex (offspring level). At the parental level, TGP can be induced in offspring by the maternal environment, the paternal environment, or both (e.g., Bonduriansky and Head, 2007; Triggs and Knell, 2012; Guillaume et al., 2016) (left panel on **Figure 4**). Parental cues can lead to (1) different information allowing offspring to trigger different responses in terms of strength or direction or at different developmental stages (multiple messages hypothesis), or (2) similar information allowing offspring to only respond to cues when they receive information through more than one modality (threshold hypothesis) or just to have a backup (backup hypothesis) (Bell and Hellmann, 2019). At the offspring level, the parental environment can impact only daughters, only sons, or both daughters and sons, which can lead to different responses in terms of strength and/or direction (review in Glover and Hill, 2012 for the effects of parental stress) (right panel on **Figure 4**). What favors sex-specific patterns at parental and offspring levels remains unexplored, but potentially has strong evolutionary implications (Bell and Hellmann, 2019).

Parental Sex-Specific Transgenerational Plasticity

It has long been assumed that induction of TGP is mostly driven by the maternal environment (Crean and Bonduriansky, 2014). Consequently, sex-specificity of TGP at the parental level has been largely overlooked. In the context of predator-induced TGP, it has only been investigated in two studies on sticklebacks (Hellmann et al., 2019; Lehto and Tinghitella,

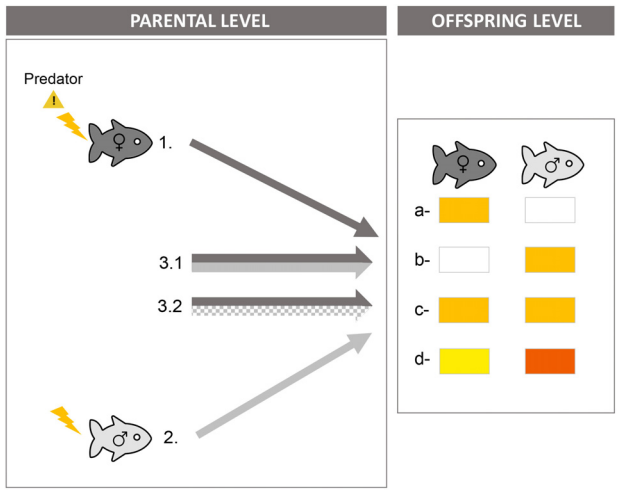

FIGURE 4 | Sex-specific predator-induced transgenerational plasticity. Even if both parents detected cues of predator presence, offspring may respond only to maternal cues (pattern 1), only to paternal cues (2) or to both parental cues with similar (3.1) or different effects on strength and/or direction between maternal and paternal cues (3.2). Furthermore, the response to parental cues may be expressed in daughters only (pattern a), in sons only (b) or in both daughters and sons but with similar (c) or different responses in terms of strength and/or direction (d).

2019). In these studies, parental sex-specific TGP patterns were highly dependent on offspring traits. Some offspring traits were affected only by maternal exposure to predator-cues (cautiousness) or only by paternal exposure (activity) (patterns 1 and 2 on **Figure 4**). For the other traits (survival, cortisol level in the daughter's egg), effects of paternal exposure interacted with effects of maternal exposure, adding yet another layer of complexity. In other words, the presence and pattern of paternal effects depended on maternal exposure to predator-cues (and vice-versa). For example, in Hellmann et al. (2019), paternal exposure decreased offspring survival to a real predator, but only when mothers had not been exposed to predator-cues. When mothers had been exposed, paternal exposure did not affect offspring survival (multiple message hypothesis). In Lehto and Tinghitella (2019), only exposure of both parents increased cortisol level of daughter's egg, while paternal exposure or maternal exposure alone had no effect (threshold hypothesis).

Offspring Sex-Specific Transgenerational Plasticity

In the context of predator-induced TGP, sex-specific TGP at the offspring level has been investigated in seven studies: three on great tits (Coslovsky and Richner, 2011; Basso and Richner, 2015a,b), two on sticklebacks (Stein and Bell, 2014; Hellmann et al., 2019), one on spider mites (Li and Zhang, 2019) and one on mice (St-Cyr and McGowan, 2015). As at the parental level, sex-specific TGP patterns were highly dependent on offspring traits. Most traits (e.g., weight, body size, survival, cautiousness) did not show any sex-specific pattern: daughters and sons were equally affected by the parental environment (Coslovsky and Richner, 2011; Stein and Bell, 2014; Basso and Richner, 2015a,b; Hellmann et al., 2019) (pattern c in **Figure 4**). For the other traits, parental exposure influenced only one sex and not the other (patterns a and b in **Figure 4**: Coslovsky and Richner, 2011; Basso and Richner, 2015a; Hellmann et al., 2019; Li and Zhang, 2019). For instance, paternal exposure to predator-cues increased activity of sons but not daughters—this could be adaptive, as only males may benefit from higher activity under predation risk in sticklebacks. Only one study showed that the paternal environment can influence both daughters and sons but with different strength: daughters increased their anti-predator behavior and cortisol level more than sons following maternal exposure to predator-cues in mice (pattern d in **Figure 4**; St-Cyr and McGowan, 2015).

At the parental level, as all results are on sticklebacks, more species remain to be described to generalize the existence and patterns of parental sex-specific TGP. However, these few results suggest that offspring integrate cues from both parents, raising questions about how parental cues combine together (i.e., whether they are additive, non-additive, repetitious, etc. Bell and Hellmann, 2019). At the offspring level, daughters and sons tend to react similarly, but when sex-specific TGP is present, transgenerational responses are often observed in one sex and not the other.

In conclusion, all these results suggest that sex of both offspring and parents may shape how TGP impacts predator-induced traits. We still do not know what favors sex-specific TGP at both parental and offspring levels. Sexual conflict may play a role if males and females have different phenotypic optima when facing predation risk (Christe et al., 2006; Meuthen et al., 2018; Burke et al., 2019). Sex-differences in ecology, gamete dispersal or offspring investment may also play a role if, for example, maternal and paternal environments predict offspring environment differently (§3.2; Bell and Hellmann, 2019).

Adjustment of Transgenerational Plasticity to Predation Risk

In the context of WGP, anti-predator defenses have long been considered as polyphenism between defended and undefended morphologies (**Figure 5A**; Harvell, 1990). For instance, a field experiment on the acorn barnacle *Chthamalus anisopoma* showed polyphenism with two distinct shell morphologies (straight *vs.* curved shape) in response to predation (Lively, 1986). In reality, however, level of expression of anti-predator defenses is often correlated with intensity of current predation risk (i.e., phenotypic modulation; **Figure 5B**; e.g., Tollrian, 1993; Van Buskirk and Arioli, 2002; Laurila et al., 2004), allowing prey to be protected from predators while limiting the costs of over-expression of defense (Tollrian et al., 2015). For example, Teplitsky et al. (2005) found a positive correlation between morphological changes and fish predator density in *Rana dalmatina* tadpoles, and Yin et al. (2015) found a positive correlation between spine development and concentration of predator-cues in the rotifer *B. calyciflorus*. We can therefore reasonably

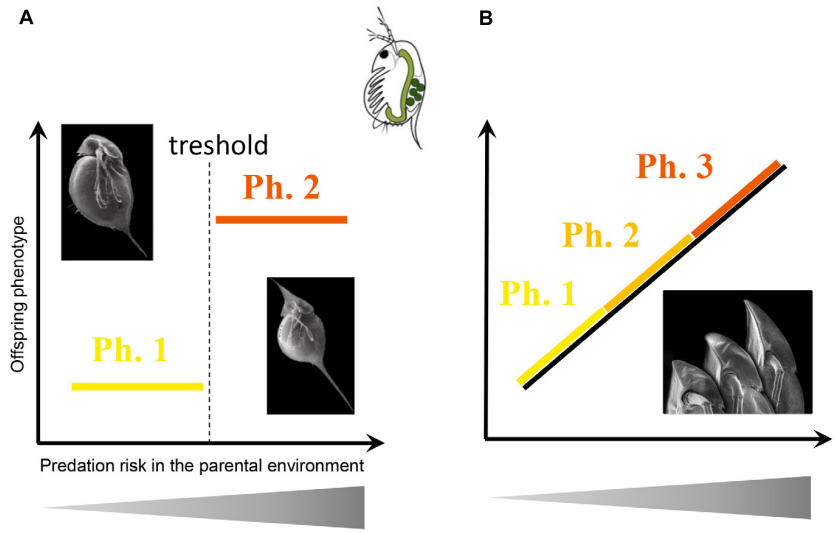

FIGURE 5 | Offspring trait expression according to increasing predation risk in the parental environment. When the risk increases, **(A)** there is no interest to be half protected against a predator, the expected response is a polyphenism (threshold response), or **(B)** the strength of the response is expected to be proportional to actual risk (phenotypic modulation). Photographs are from Agrawal (2001), (reprinted with permission from AAAS) and from Herzog and Laforsch (2013).

suggest that the level of TGP (i.e., level of expression of offspring defenses) should also be proportional to the level of predation risk perceived by ancestors (**Figure 5B**). This hypothesis has been investigated in only three papers. Podjasek et al. (2005) clearly found a positive and relatively linear correlation between production of winged offspring in the pea aphid *A. pisum* and concentration of alarm pheromones, a reliable cue of predation risk. Also in the pea aphid, Kunert and Weisser (2003) found a non-linear relation between transgenerational response and parental predation risk, with the strongest response (i.e., highest percentage of winged offspring) being observed at an intermediate predation risk and the lowest response at very high and very low predation risk. Finally, Freinschlag and Schausberger (2016) did not find any correlation between developmental time or anti-predator behavior of juvenile spider mites (*T. urticae*) and the intensity of predation risk (no, low, moderate, severe) experienced by their mothers. To get a consistent picture, more empirical studies are needed on how the intensity of parental predation risk modulates the expression level of offspring defenses.

CONCLUSION

Predator-prey interactions have long been a focus of ecological and evolutionary studies, likely because almost all species are engaged in such interactions. The literature has accumulated a solid knowledge of within-generation plasticity (WGP) in prey and predators, and it is commonly observed that prey develop defensive phenotypes when they detect predation risk. Over the past two decades, some studies have also shown that prey exposed to predator-cues can produce offspring with better defenses than offspring from predator-free parents [transgenerational plasticity (TGP)]. This review summarizes current knowledge on predator-induced TGP in metazoans. Most of the 55 studies we reviewed focused on five model taxa: fish (24%) aphids (16%), water fleas (15%), aquatic snails (15%), and birds (11%). Although a more diverse set of taxa would allow for more robust generalizations, the study of TGP requires rearing animals over at least two generations, which limits the study to taxa with short generation times. This explains why some taxa that are widely used to study predator-induced WGP, such as amphibians, are still lacking in TGP studies.

Our review highlights that all kinds of traits are prone to exhibit predator-induced TGP, even the most labile ones such as behavior. Predator-induced TGP seems to confer increased fitness in most studies, is evolvable (e.g., shows signatures of local adaptation) and can involve epigenetic mechanisms of inheritance, although these aspects are still too rarely evaluated. However, predator-induced TGP is often characterized by complex phenotypic patterns that can be difficult to interpret and do not always fit with simple explanatory scenarios invoking clear adaptive advantages. The induction, strength, and direction of predator-induced TGP depend on several factors and their interplay:

(1) Timing is important, but no general sensitive or critical periods can be defined, either in terms of parental information or offspring expression.
(2) Predator-induced TGP can persist for more than one generation (three generations on average in studies conducted over a sufficiently long time-frame), meaning that past environments may interact with each other and with offspring environmental conditions in shaping the anti-predator phenotype.

(3) The induction of TGP can depend on which parent (mother or father) has been exposed to predation risk, and its expression can be different in daughters and sons.
(4) While prey are often able to adjust their within-generational defenses to the level of predation risk, evidence of such scaling is lacking for TGP, but the number of relevant studies is still very limited.

Despite these general conclusions, we are only at the beginning of understanding the processes involved in predator-induced TGP. Indeed, most current studies only describe the existence of TGP in response to predation risk, while very few unravel the underlying processes. This makes TGP an exciting and challenging research topic for future studies. Such studies will be necessary (1) to add more examples of predator-induced TGP, (2) to study TGP in different offspring environments to account for the interplay between past and current predation risk, and (3) to disentangle the complexity of TGP (i.e., dissect processes and underlying mechanisms of induction and expression) in order to evaluate its adaptive value and its ecological and evolutionary impacts in predator-prey interactions. A relevant perspective would be to consider implications of TGP at the population level. Particularly, how predator-induced TGP may buffer the top-down effects of predators on prey population size, allowing better prediction of population dynamics of predator and prey and dynamics of food webs in general.

AUTHOR CONTRIBUTIONS

All authors have contributed equally to the work, and approved it for publication.

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REFERENCES

Agrawal, A. A. (2001). Phenotypic plasticity in the interactions and evolution of species. *Science* 294, 321–326. doi: 10.1126/science.1060701

Agrawal, A. A., Laforsch, C., and Tollrian, R. (1999). Transgenerational induction of defences in animals and plants. *Nature* 401, 60–63. doi: 10.1038/43425

Auge, G. A., Leverett, L. D., Edwards, B. R., and Donohue, K. (2017). Adjusting phenotypes via within- and across-generational plasticity. *New Phytol.* 216, 343–349. doi: 10.1111/nph.14495

Auld, J. R., Agrawal, A. A., and Relyea, R. A. (2010). Re-evaluating the costs and limits of adaptive phenotypic plasticity. *Proc. R. Soc. B Biol. Sci.* 277, 503–511. doi: 10.1098/rspb.2009.1355

Auld, J. R., and Relyea, R. A. (2011). Adaptive plasticity in predator-induced defenses in a common freshwater snail: altered selection and mode of predation due to prey phenotype. *Evol. Ecol.* 25, 189–202. doi: 10.1007/s10682-010-9394-1

Basso, A., Coslovsky, M., and Richner, H. (2014). Parasite- and predator-induced maternal effects in the great tit (*Parus major*). *Behav. Ecol.* 25, 1105–1114. doi: 10.1093/beheco/aru088

Basso, A., and Richner, H. (2015a). Effects of nest predation risk on female incubation behavior and offspring growth in great tits. *Behav. Ecol. Sociobiol.* 69, 977–989. doi: 10.1007/s00265-015-1910-4

Basso, A., and Richner, H. (2015b). Predator-specific effects on incubation behaviour and offspring growth in great tits. *PLoS One* 10:e0121088. doi: 10.1371/journal.pone.0121088

Beaman, J. E., White, C. R., and Seebacher, F. (2016). Evolution of plasticity: mechanistic link between development and reversible acclimation. *Trends Ecol. Evol.* 31, 237–249. doi: 10.1016/j.tree.2016.01.004

Beaty, L. E., Wormington, J. D., Kensinger, B. J., Bayley, K. N., Goeppner, S. R., Gustafson, K. D., et al. (2016). Shaped by the past, acting in the present: transgenerational plasticity of anti-predatory traits. *Oikos* 125, 1570–1576. doi: 10.1111/oik.03114

Bell, A. M., and Hellmann, J. (2019). An integrative framework for understanding the mechanisms and multigenerational consequences of transgenerational plasticity. *Annu. Rev. Ecol. Evol. Syst.* 50, 97–118. doi: 10.1146/annurev-ecolsys-110218-024613

Bestion, E., Teyssier, A., Aubret, F., Clobert, J., and Cote, J. (2014). Maternal exposure to predator scents: offspring phenotypic adjustment and dispersal. *Proc. R. Soc. B Biol. Sci.* 281:20140701. doi: 10.1098/rspb.2014.0701

Bonduriansky, R., and Head, M. (2007). Maternal and paternal condition effects on offspring phenotype in *Telostylinus angusticollis* (Diptera: Neriidae). *J. Evol. Biol.* 20, 2379–2388. doi: 10.1111/j.1420-9101.2007.01419.x

Boškoviæ, A., and Rando, O. J. (2018). Transgenerational Epigenetic Inheritance. *Annu. Rev. Genet.* 52, 21–41. doi: 10.1146/annurev-genet-120417-031404

Burke, N. W., Nakagawa, S., and Bonduriansky, R. (2019). Sexual conflict explains diverse patterns of transgenerational plasticity. *bioRxiv* [Preprint]. doi: 10.1101/846287

Burton, T., and Metcalfe, N. B. (2014). Can environmental conditions experienced in early life influence future generations? *Proc. R. Soc. B Biol. Sci.* 281:20140311. doi: 10.1098/rspb.2014.0311

Christe, P., Keller, L., and Roulin, A. (2006). The predation cost of being a male: implications for sex-specific rates of ageing. *Oikos* 114, 381–384. doi: 10.1111/j.2006.0030-1299.15130.x

Coslovsky, M., and Richner, H. (2011). Predation risk affects offspring growth via maternal effects: maternal effects due to predation risk. *Funct. Ecol.* 25, 878–888. doi: 10.1111/j.1365-2435.2011.01834.x

Coslovsky, M., and Richner, H. (2012). Preparing offspring for a dangerous world: potential costs of being wrong. *PLoS One* 7:e48840. doi: 10.1371/journal.pone.0048840

Coutellier, L., and Würbel, H. (2009). Early environmental cues affect object recognition memory in adult female but not male C57BL/6 mice. *Behav. Brain Res.* 203, 312–315. doi: 10.1016/j.bbr.2009.05.001

Crean, A. J., and Bonduriansky, R. (2014). What is a paternal effect? *Trends Ecol. Evol.* 29, 554–559. doi: 10.1016/j.tree.2014.07.009

de Vries, H., and MacDougal, D. T. (1905). *Species and Varieties, Their Origin by Mutation: Lectures Delivered at the University of California*. Chicago, IL: The Open court publishing company.

DeWitt, T. J., Sih, A., and Wilson, D. S. (1998). Costs and limits of phenotypic plasticity. *Trends Ecol. Evol.* 13, 77–81. doi: 10.1016/S0169-5347(97)01274-3

Dingemanse, N. J., and Wolf, M. (2013). Between-individual differences in behavioural plasticity within populations: causes and consequences. *Anim. Behav.* 85, 1031–1039. doi: 10.1016/j.anbehav.2012.12.032

Dixon, A. F. G., and Agarwala, B. K. (1999). Ladybird-induced life–history changes in aphids. *Proc. R. Soc. Lond. B Biol. Sci.* 266, 1549–1553. doi: 10.1098/rspb.1999.0814

Donelan, S. C., Hellmann, J. K., Bell, A. M., Luttbeg, B., Sheriff, M. J., and Sih, A. (2020). Transgenerational plasticity in human-altered environments. *Trends Ecol. Evol.* 35, 115–124. doi: 10.1016/j.tree.2019.09.003

Donelan, S. C., and Trussell, G. C. (2015). Parental effects enhance risk tolerance and performance in offspring. *Ecology* 96, 2049–2055. doi: 10.1890/14-1773.1

Donelan, S. C., and Trussell, G. C. (2018a). Parental and embryonic experiences with predation risk affect prey offspring behaviour and performance. *Proc. R. Soc. B Biol. Sci.* 285:20180034. doi: 10.1098/rspb.2018.0034

Donelan, S. C., and Trussell, G. C. (2018b). Synergistic effects of parental and embryonic exposure to predation risk on prey offspring size at emergence. *Ecology* 99, 68–78. doi: 10.1002/ecy.2067

Donelson, J. M., Salinas, S., Munday, P. L., and Shama, L. N. S. (2018). Transgenerational plasticity and climate change experiments: where do we go from here? *Glob. Change Biol.* 24, 13–34. doi: 10.1111/gcb.13903

Duempelmann, L., Skribbe, M., and Bühler, M. (2020). Small RNAs in the transgenerational inheritance of epigenetic information. *Trends Genet.* 36, 203–214. doi: 10.1016/j.tig.2019.12.001

Dzialowski, A. R., Lennon, J. T., O'Brien, W. J., and Smith, V. H. (2003). Predator-induced phenotypic plasticity in the exotic cladoceran Daphnia lumholtzi. *Freshw. Biol.* 48, 1593–1602. doi: 10.1046/j.1365-2427.2003.01111.x

Eggers, S., Griesser, M., Nystrand, M., and Ekman, J. (2006). Predation risk induces changes in nest-site selection and clutch size in the Siberian jay. *Proc. R. Soc. B Biol. Sci.* 273, 701–706. doi: 10.1098/rspb.2005.3373

Evans, J. P., Gasparini, C., and Pilastro, A. (2007). Female guppies shorten brood retention in response to predator cues. *Behav. Ecol. Sociobiol.* 61, 719–727. doi: 10.1007/s00265-006-0302-1

Ezard, T. H. G., Prizak, R., and Hoyle, R. B. (2014). The fitness costs of adaptation via phenotypic plasticity and maternal effects. *Funct. Ecol.* 28, 693–701. doi: 10.1111/1365-2435.12207

Fallet, M., Luquet, E., David, P., and Cosseau, C. (2020). Epigenetic inheritance and intergenerational effects in mollusks. *Gene* 729:144166. doi: 10.1016/j.gene.2019.144166

Fawcett, T. W., and Frankenhuis, W. E. (2015). Adaptive explanations for sensitive windows in development. *Front. Zool.* 12:S3. doi: 10.1186/1742-9994-12-S1-S3

Freinschlag, J., and Schausberger, P. (2016). Predation risk-mediated maternal effects in the two-spotted spider mite, *Tetranychus urticae*. *Exp. Appl. Acarol.* 69, 35–47. doi: 10.1007/s10493-016-0014-9

Ghalambor, C., Angeloni, L. M., and Carroll, S. (2010). "Behavior as phenotypic plasticity," in *Evolutionary Behavioral Ecology*, eds C. Fox and D. Westneat (Oxford: Oxford University Press), 90–107.

Giesing, E. R., Suski, C. D., Warner, R. E., and Bell, A. M. (2011). Female sticklebacks transfer information via eggs: effects of maternal experience with predators on offspring. *Proc. R. Soc. B Biol. Sci.* 278, 1753–1759. doi: 10.1098/rspb.2010.1819

Gilbert, J. J. (1966). Rotifer ecology and embryological induction. *Science* 151, 1234–1237. doi: 10.1126/science.151.3715.1234

Glover, V., and Hill, J. (2012). Sex differences in the programming effects of prenatal stress on psychopathology and stress responses: An evolutionary perspective. *Physiol. Behav.* 106, 736–740. doi: 10.1016/j.physbeh.2012.02.011

Goeppner, S. R., Roberts, M. E., Beaty, L. E., and Luttbeg, B. (2020). Freshwater snail responses to fish predation integrate phenotypic plasticity and local adaptation. *Aquat. Ecol.* 54, 309–322. doi: 10.1007/s10452-019-09744-x

Guillaume, A. S., Monro, K., and Marshall, D. J. (2016). Transgenerational plasticity and environmental stress: do paternal effects act as a conduit or a buffer? *Funct. Ecol.* 30, 1175–1184. doi: 10.1111/1365-2435.12604

Hales, N. R., Schield, D. R., Andrew, A. L., Card, D. C., Walsh, M. R., and Castoe, T. A. (2017). Contrasting gene expression programs correspond with predator-induced phenotypic plasticity within and across generations in *Daphnia*. *Mol. Ecol.* 26, 5003–5015. doi: 10.1111/mec.14213

Harvell, C. D. (1990). The ecology and evolution of inducible defenses. *Q. Rev. Biol.* 65, 323–340. doi: 10.1086/416841

Hellmann, J. K., Bukhari, S. A., Deno, J., and Bell, A. M. (2019). Sex-specific transgenerational plasticity in threespined sticklebacks. *bioRxiv* [Preprint]. doi: 10.1101/763862

Hellmann, J. K., Carlson, E. R., and Bell, A. M. (2020). Sex-specific transgenerational plasticity II: Grandpaternal effects are lineage- and sex-specific in threespined sticklebacks. *bioRxiv* 796995. doi: 10.1101/796995

Herzog, Q., and Laforsch, C. (2013). Modality matters for the expression of inducible defenses: introducing a concept of predator modality. *BMC Biol.* 11:113. doi: 10.1186/1741-7007-11-113

Hunter, L. T. B., and Skinner, J. D. (1998). Vigilance behaviour in african ungulates: the role of predation pressure. *Behaviour* 135, 195–211. doi: 10.1163/156853998793066320

Jacobs, J. (1967). Untersuchungen zur Funktion and Evolution der Zyklomorphose bei *Daphnia*, mit besonderer Berucksichtigung der Selecktion durch Fischc. *Arch. Hydrobiol.* 62, 467–541.

Keiser, C. N., and Mondor, E. B. (2013). Transgenerational behavioral plasticity in a parthenogenetic insect in response to increased predation risk. *J. Insect Behav.* 26, 603–613. doi: 10.1007/s10905-013-9376-6

Kishida, O., Trussell, G. C., Mougi, A., and Nishimura, K. (2010). Evolutionary ecology of inducible morphological plasticity in predator–prey interaction: toward the practical links with population ecology. *Popul. Ecol.* 52, 37–46. doi: 10.1007/s10144-009-0182-0

Kopp, M., and Tollrian, R. (2003). Reciprocal phenotypic plasticity in a predator–prey system: inducible offences against inducible defences? *Ecol. Lett.* 6, 742–748. doi: 10.1046/j.1461-0248.2003.00485.x

Kuijper, B., and Hoyle, R. B. (2015). When to rely on maternal effects and when on phenotypic plasticity? *Evolution* 69, 950–968. doi: 10.1111/evo.12635

Kunert, G., and Weisser, W. W. (2003). The interplay between density- and trait-mediated effects in predator-prey interactions: a case study in aphid wing polymorphism. *Oecologia* 135, 304–312. doi: 10.1007/s00442-003-1185-8

Kunert, G., and Weisser, W. W. (2005). The importance of antennae for pea aphid wing induction in the presence of natural enemies. *Bull. Entomol. Res.* 95, 125–131. doi: 10.1079/BER2004342

Laurila, A., Järvi-Laturi, M., Pakkasmaa, S., and Merilä, J. (2004). Temporal variation in predation risk: stage-dependency, graded responses and fitness costs in tadpole antipredator defences. *Oikos* 107, 90–99. doi: 10.1111/j.0030-1299.2004.13126.x

Lehto, W. R., and Tinghitella, R. M. (2019). Joint maternal and paternal stress increases the cortisol in their daughters' eggs. *Evol. Ecol. Res.* 20, 133–144.

Leimar, O., and McNamara, J. M. (2015). The evolution of transgenerational integration of information in heterogeneous environments. *Am. Nat.* 185, E55–E69. doi: 10.1086/679575

Li, D., and Lee, W. S. (2004). Predator-induced plasticity in web-building behaviour. *Anim. Behav.* 67, 309–318. doi: 10.1016/j.anbehav.2003.06.011

Li, G.-Y., and Zhang, Z.-Q. (2019). Development, lifespan and reproduction of spider mites exposed to predator-induced stress across generations. *Biogerontology* 20, 871–882. doi: 10.1007/s10522-019-09835-0

Lima, S. L. (1998). Nonlethal effects in the ecology of predator-prey interactions. what are the ecological effects of anti-predator decision-making?. *Bioscience* 48, 25–34. doi: 10.2307/1313225

Lively, C. M. (1986). Predator-induced shell dimorphism in the acorn barnacle chthamalus anisopoma. *Evolution* 40, 232–242. doi: 10.1111/j.1558-5646.1986.tb00466.x

Luquet, E., and Tariel, J. (2016). Offspring reaction norms shaped by parental environment: interaction between within- and trans-generational plasticity of inducible defenses. *BMC Evol. Biol.* 16:209. doi: 10.1186/s12862-016-0795-9

McGhee, K. E., and Bell, A. M. (2014). Paternal care in a fish: epigenetics and fitness enhancing effects on offspring anxiety. *Proc. R. Soc. B Biol. Sci.* 281:20141146. doi: 10.1098/rspb.2014.1146

McGhee, K. E., Pintor, L. M., Suhr, E. L., and Bell, A. M. (2012). Maternal exposure to predation risk decreases offspring antipredator behaviour and survival in threespined stickleback. *Funct. Ecol.* 26, 932–940. doi: 10.1111/j.1365-2435.2012.02008.x

Meuthen, D., Baldauf, S. A., Bakker, T. C. M., and Thünken, T. (2018). Neglected patterns of variation in phenotypic plasticity: age- and sex-specific antipredator plasticity in a cichlid fish. *Am. Nat.* 191, 475–490. doi: 10.1086/696264

Mikulski, A., and Pijanowska, J. (2010). When and how can *Daphnia* prepare their offspring for the threat of predation? *Hydrobiologia* 643, 21–26. doi: 10.1007/s10750-010-0131-0

Mitchell, M. D., Bairos-Novak, K. R., and Ferrari, M. C. O. (2017). Mechanisms underlying the control of responses to predator odours in aquatic prey. *J. Exp. Biol.* 220, 1937–1946. doi: 10.1242/jeb.135137

Miyakawa, H., Imai, M., Sugimoto, N., Ishikawa, Y., Ishikawa, A., Ishigaki, H., et al. (2010). Gene up-regulation in response to predator kairomones in the water flea, *Daphnia pulex*. *BMC Dev. Biol.* 10:45. doi: 10.1186/1471-213X-10-45

Mommer, B. C., and Bell, A. M. (2013). A test of maternal programming of offspring stress response to predation risk in threespine sticklebacks. *Physiol. Behav.* 122, 222–227. doi: 10.1016/j.physbeh.2013.04.004

Mondor, E. B., Rosenheim, J. A., and Addicott, J. F. (2005). Predator-induced transgenerational phenotypic plasticity in the cotton aphid. *Oecologia* 142, 104–108. doi: 10.1007/s00442-004-1710-4

Monteforte, S., Cattelan, S., Morosinotto, C., Pilastro, A., and Grapputo, A. (2020). Maternal predator-exposure affects offspring size at birth but not telomere length in a live-bearing fish. *Ecol. Evol.* 10, 2030–2039. doi: 10.1002/ece3.6035

Morales, J., Lucas, A., and Velando, A. (2018). Maternal programming of offspring antipredator behavior in a seabird. *Behav. Ecol.* 29, 479–485. doi: 10.1093/beheco/arx197

Murren, C. J., Auld, J. R., Callahan, H., Ghalambor, C. K., Handelsman, C. A., Heskel, M. A., et al. (2015). Constraints on the evolution of phenotypic plasticity: limits and costs of phenotype and plasticity. *Heredity* 115, 293–301. doi: 10.1038/hdy.2015.8

Nicoglou, A. (2018). "The concept of plasticity in the history of the nature-nurture debate in the early twentieth century," in *The Palgrave Handbook of Biology and Society*, eds M. Meloni, J. Cromby, D. Fitzgerald, and S. Lloyd (London: Palgrave Macmillan), 97–122. doi: 10.1057/978-1-137-52879-7_5

Norouzitallab, P., Baruah, K., Vanrompay, D., and Bossier, P. (2019). Can epigenetics translate environmental cues into phenotypes? *Sci. Total Environ.* 647, 1281–1293. doi: 10.1016/j.scitotenv.2018.08.063

Padilla, D. (2001). Food and environmental cues trigger an inducible offence. *Evol. Ecol. Res.* 3, 15–25.

Piersma, T., and Drent, J. (2003). Phenotypic flexibility and the evolution of organismal design. *Trends Ecol. Evol.* 18, 228–233. doi: 10.1016/S0169-5347(03)00036-3

Pigliucci, M. (2005). Evolution of phenotypic plasticity: Where are we going now? *Trends Ecol. Evol.* 20, 481–486. doi: 10.1016/j.tree.2005.06.001

Plautz, S. C., Guest, T., Funkhouser, M. A., and Salice, C. J. (2013). Transgenerational cross-tolerance to stress: parental exposure to predators increases offspring contaminant tolerance. *Ecotoxicology* 22, 854–861. doi: 10.1007/s10646-013-1056-y

Podjasek, J. O., Bosnjak, L. M., Brooker, D. J., and Mondor, E. B. (2005). Alarm pheromone induces a transgenerational wing polyphenism in the pea aphid, *Acyrthosiphon pisum*. *Can. J. Zool.* 83, 1138–1141. doi: 10.1139/z05-108

Prizak, R., Ezard, T. H. G., and Hoyle, R. B. (2014). Fitness consequences of maternal and grandmaternal effects. *Ecol. Evol.* 4, 3139–3145. doi: 10.1002/ece3.1150

Riessen, H. P. (1999). Predator-induced life history shifts in *Daphnia*: a synthesis of studies using meta-analysis. *Can. J. Fish. Aquat. Sci.* 56, 2487–2494. doi: 10.1139/f99-155

Roche, D. P., McGhee, K. E., and Bell, A. M. (2012). Maternal predator-exposure has lifelong consequences for offspring learning in threespined sticklebacks. *Biol. Lett.* 8, 932–935. doi: 10.1098/rsbl.2012.0685

Salinas, S., Brown, S. C., Mangel, M., and Munch, S. B. (2013). Non-genetic inheritance and changing environments. *Non Genet. Inherit.* 1, 38–50. doi: 10.2478/ngi-2013-0005

Schield, D. R., Walsh, M. R., Card, D. C., Andrew, A. L., Adams, R. H., and Castoe, T. A. (2016). EpiRADseq: scalable analysis of genomewide patterns of methylation using next-generation sequencing. *Methods Ecol. Evol.* 7, 60–69. doi: 10.1111/2041-210X.12435

Segers, F. H. I. D., Gerber, B., and Taborsky, B. (2011). Do maternal food deprivation and offspring predator cues interactively affect maternal effort in fish? *Ethology* 117, 708–721. doi: 10.1111/j.1439-0310.2011.01922.x

Segers, F. H. I. D., and Taborsky, B. (2012). Juvenile exposure to predator cues induces a larger egg size in fish. *Proc. R. Soc. B Biol. Sci.* 279, 1241–1248. doi: 10.1098/rspb.2011.1290

Seiter, M., and Schausberger, P. (2015). Maternal intraguild predation risk affects offspring anti-predator behavior and learning in mites. *Sci. Rep.* 5:15046. doi: 10.1038/srep15046

Sentis, A., Bertram, R., Dardenne, N., Ramon-Portugal, F., Espinasse, G., Louit, I., et al. (2018). Evolution without standing genetic variation: change in transgenerational plastic response under persistent predation pressure. *Heredity* 121, 266–281. doi: 10.1038/s41437-018-0108-8

Sentis, A., Bertram, R., Dardenne, N., Ramon-Portugal, F., Louit, I., Le Trionnaire, G., et al. (2019). Different phenotypic plastic responses to predators observed among aphid lineages specialized on different host plants. *Sci. Rep.* 9:9017. doi: 10.1038/s41598-019-45220-0

Sentis, A., Hemptinne, J.-L., and Brodeur, J. (2017). Non-additive effects of simulated heat waves and predators on prey phenotype and transgenerational phenotypic plasticity. *Glob. Change Biol.* 23, 4598–4608. doi: 10.1111/gcb.13674

Sheriff, M. J., Krebs, C. J., and Boonstra, R. (2009). The sensitive hare: sublethal effects of predator stress on reproduction in snowshoe hares. *J. Anim. Ecol.* 78, 1249–1258. doi: 10.1111/j.1365-2656.2009.01552.x

Sheriff, M. J., Krebs, C. J., and Boonstra, R. (2010). The ghosts of predators past: population cycles and the role of maternal programming under fluctuating predation risk. *Ecology* 91, 2983–2994. doi: 10.1890/09-1108.1

Shine, R., and Downes, S. J. (1999). Can pregnant lizards adjust their offspring phenotypes to environmental conditions? *Oecologia* 119, 1–8. doi: 10.1007/s004420050754

Sih, A., Ziemba, R., and Harding, K. C. (2000). New insights on how temporal variation in predation risk shapes prey behavior. *Trends Ecol. Evol.* 15, 3–4. doi: 10.1016/S0169-5347(99)01766-8

Sogard, S. M. (1997). Size-selective mortality in the juvenile stage of teleost fishes: a review. *Bull. Mar. Sci.* 60, 1129–1157.

St-Cyr, S., and McGowan, P. O. (2015). Programming of stress-related behavior and epigenetic neural gene regulation in mice offspring through maternal exposure to predator odor. *Front. Behav. Neurosci.* 9:145. doi: 10.3389/fnbeh.2015.00145

Stein, L. R., and Bell, A. M. (2014). Paternal programming in sticklebacks. *Anim. Behav.* 95, 165–171. doi: 10.1016/j.anbehav.2014.07.010

Stein, L. R., Bukhari, S. A., and Bell, A. M. (2018). Personal and transgenerational cues are nonadditive at the phenotypic and molecular level. *Nat. Ecol. Evol.* 2, 1306–1311. doi: 10.1038/s41559-018-0605-4

Steiner, U. K., and Pfeiffer, T. (2007). Optimizing time and resource allocation trade-offs for investment into morphological and behavioral defense. *Am. Nat.* 169, 118–129. doi: 10.1086/509939

Storm, J. J., and Lima, S. L. (2010). Mothers forewarn offspring about predators: a transgenerational maternal effect on behavior. *Am. Nat.* 175, 382–390. doi: 10.1086/650443

Stratmann, A., and Taborsky, B. (2014). Antipredator defences of young are independently determined by genetic inheritance, maternal effects and own early experience in mouthbrooding cichlids. *Funct. Ecol.* 28, 944–953. doi: 10.1111/1365-2435.12224

Svanbäck, R., Pineda-Krch, M., and Doebeli, M. (2009). Fluctuating population dynamics promotes the evolution of phenotypic plasticity. *Am. Nat* 174, 176–189. doi: 10.1086/600112

Tariel, J., Plénet, S., and Luquet, É (2019). How past exposure to predation affects personality and behavioural plasticity? *bioRxiv* [Preprint]. doi: 10.1101/844258

Tariel, J., Plénet, S., and Luquet, É (2020). Transgenerational plasticity of inducible defences: combined effects of grand-parental, parental and current environments. *Ecol. Evol.* 10, 2367–2376. doi: 10.1002/ece3.6046

Teplitsky, C., Plenet, S., Léna, J.-P., Mermet, N., Malet, E., and Joly, P. (2005). Escape behaviour and ultimate causes of specific induced defences in an anuran tadpole. *J. Evol. Biol.* 18, 180–190. doi: 10.1111/j.1420-9101.2004.00790.x

Tigreros, N., Norris, R. H., and Thaler, J. S. (2019). Maternal effects across life stages: larvae experiencing predation risk increase offspring provisioning. *Ecol. Entomol.* 44, 738–744. doi: 10.1111/een.12752

Tollrian, R. (1993). Neckteeth formation in *Daphnia pulex* as an example of continuous phenotypic plasticity: morphological effects of Chaoborus kairomone concentration and their quantification. *J. Plankton Res.* 15, 1309–1318. doi: 10.1093/plankt/15.11.1309

Tollrian, R. (1995). Predator-induced morphological defenses: costs, life history shifts, and maternal effects in Daphnia Pulex. *Ecology* 76, 1691–1705. doi: 10.2307/1940703

Tollrian, R., Duggen, S., Weiss, L. C., Laforsch, C., and Kopp, M. (2015). Density-dependent adjustment of inducible defenses. *Sci. Rep.* 5:12736. doi: 10.1038/srep12736

Tollrian, R., and Harvell, C. D. (eds) (1999). *The Ecology and Evolution of Inducible Defenses*. Princeton, NJ: Princeton University Press.

Tollrian, R., and Leese, F. (2010). Ecological genomics: steps towards unraveling the genetic basis of inducible defenses in Daphnia. *BMC Biol.* 8:51. doi: 10.1186/1741-7007-8-51

Torres-Dowdall, J., Handelsman, C. A., Reznick, D. N., and Ghalambor, C. K. (2012). Local adaptation and the evolution of phenotypic plasticity in Trinidadian Guppies (poecilia Reticulata). *Evolution* 66, 3432–3443. doi: 10.1111/j.1558-5646.2012.01694.x

Triggs, A. M., and Knell, R. J. (2012). Parental diet has strong transgenerational effects on offspring immunity. *Funct. Ecol.* 26, 1409–1417. doi: 10.1111/j.1365-2435.2012.02051.x

Uller, T. (2008). Developmental plasticity and the evolution of parental effects. *Trends Ecol. Evol.* 23, 432–438. doi: 10.1016/j.tree.2008.04.005

Van Buskirk, J. (2002). A Comparative test of the adaptive plasticity hypothesis: relationships between habitat and phenotype in anuran larvae. *Am. Nat.* 160, 87–102. doi: 10.1086/340599

Van Buskirk, J., and Arioli, M. (2002). Dosage response of an induced defense: how sensitive are tadpoles to predation risk? *Ecology* 83, 1580–1585. doi: 10.2307/3071977

Walsh, M. R., Castoe, T., Holmes, J., Packer, M., Biles, K., Walsh, M., et al. (2016). Local adaptation in transgenerational responses to predators. *Proc. R. Soc. B Biol. Sci.* 283:20152271. doi: 10.1098/rspb.2015.2271

Walsh, M. R., Cooley, F., Biles, K., and Munch, S. B. (2015). Predator-induced phenotypic plasticity within- and across-generations: a challenge for theory? *Proc. R. Soc. B Biol. Sci.* 282:20142205. doi: 10.1098/rspb.2014.2205

Weisser, W. W., Braendle, C., and Minoretti, N. (1999). Predator-induced morphological shift in the pea aphid. *Proc. R. Soc. Lond. B Biol. Sci.* 266, 1175–1181. doi: 10.1098/rspb.1999.0760

West-Eberhard, M. J. (2003). *Developmental Plasticity and Evolution*. Oxford: Oxford University Press.

Woltereck, R. (1909). Weitere experimentelle Untersuchungen über Artveränderung, speziell über das Wesen quantitativer Artunterschiede bei Daphniden. *Vers. Dtsch. Zool. Gesellschaft* 110–172.

Yin, J., Zhou, M., Lin, Z., Li, Q. Q., and Zhang, Y. (2019). Transgenerational effects benefit offspring across diverse environments: a meta-analysis in plants and animals. *Ecol. Lett.* 22, 1976–1986. doi: 10.1111/ele.13373

Yin, X. W., Zhao, N. X., Wang, B. H., Li, W. J., and Zhang, Z. N. (2015). Transgenerational and within-generational induction of defensive morphology in *Brachionus calyciflorus* (Rotifera): importance of maternal effect. *Hydrobiologia* 742, 313–325. doi: 10.1007/s10750-014-1995-1

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Mimicking Transgenerational Signals of Future Stress: Thermal Tolerance of Juvenile Chinook Salmon Is More Sensitive to Elevated Rearing Temperature Than Exogenously Increased Egg Cortisol

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Elevated temperatures resulting from climate change are expected to disproportionately affect ectotherms given their biological function has a direct link to environmental temperature. Thus, as climate change leads to rapid increases in water temperatures in rivers, aquatic ectotherms, such as fish may be highly impacted. Organisms can respond to these stressors through flexible and rapid phenotypic change induced via developmental and/or transgenerational plasticity. In oviparous species, gravid females may translate environmental stress across generations via increased exposure of eggs to maternally derived glucocorticoids (i.e., maternal stress), which has been shown to result in diverse phenotypic effects in offspring. Recent studies suggest these phenotypic changes from maternal glucocorticoids may elicit predictive adaptive responses, where offspring exposed to maternal stress may be better prepared for the stressful environment they will encounter (i.e., environmental match hypothesis). We applied the environmental match hypothesis to examine whether a prenatal exogenous increase in egg cortisol may prepare Chinook salmon offspring (*Oncorhynchus tshawytscha*) to cope with thermal challenges after being reared in chronically elevated temperatures. Specifically, we exposed eggs to aqueous bath of cortisol-dosed (1,000 ng/mL) or control (0 ng/ml) solutions, and then raised both treatments at current (+0°C—contemporary ambient river temperature) or elevated (+3°C—projected future river temperature) thermal regimes. We quantified thermal performance in fish 7–9 month post fertilization using two methods: via critical thermal maximum (CT_{Max}), and energetic responses (in plasma cortisol, glucose, and lactate) to environmentally relevant, but challenging thermal spikes over 3 days. Overall, we found that exposure to elevated rearing temperatures had a large impact on thermal tolerance, where elevated-temperature reared offspring had significantly higher CT_{Max}. In comparison, egg cortisol

treatment had little to no clear effects on CT_{Max} and blood energetic response. Our study demonstrates the importance of elevated water temperatures as an inducer of offspring phenotypes (via early developmental cues), and highlights the significance of examining offspring performance in environments with ecologically relevant stressors.

Keywords: maternal stress, prenatal stress, thermal stress, climate change, CT_{Max}, plasma cortisol, glucose, lactate

INTRODUCTION

Climate change is a major contributor to rapid global changes, whether via increasing average temperatures (Solomon et al., 2012), or increasing frequency of extreme weather events (e.g., droughts: Rahmstorf and Coumou, 2011). Aquatic systems are expected to be highly impacted by climate change, not only through increasing average water temperatures, but by changing the hydrological cycle (e.g., increase in precipitation variation), which causes more extremes in water flow (e.g., droughts and floods), and by increasing daily temperature extremes (e.g., thermal spikes: Mantua et al., 2010; Woodward et al., 2016). Aquatic ectotherms such as reptiles, amphibians and most fish species are potentially sensitive to these alterations in temperature events, which may have lasting effects within and across generations (Deutsch et al., 2008; McCullough et al., 2009; Buckley and Huey, 2016). Indeed, juvenile ectotherms that develop in warmer temperatures have been shown to have altered phenotypes: smaller bodies (Cingi et al., 2010; Sheridan and Bickford, 2011; Whitney et al., 2014), altered immunity (Alcorn et al., 2002; Pérez-Casanova et al., 2008), and increased metabolism (Clarke and Johnston, 1999; Enders and Boisclair, 2016). These responses have been shown to impact performance (i.e., higher thermal tolerance: Bickford et al., 2010; Dillon et al., 2010; Sandblom et al., 2016, but see Chen et al., 2013) and fitness (i.e., survival: Martins et al., 2012; Rohr and Palmer, 2013).

To respond and persist within a rapidly changing world, species require mechanisms such as developmental plasticity (Hendry et al., 2008; Chevin et al., 2010), phenotypically flexible responses (Piersma and Drent, 2003; Franklin et al., 2007; Forsman, 2015), epigenetic inheritance (Lind and Spagopoulou, 2018), and contemporary evolution (Carroll et al., 2007) which act within and across generations. Environmentally induced plasticity can enable organisms to optimize growth, morphology, and physiology in response to current (or expected) environmental conditions to ultimately maximize performance, reproduction, and survival (Seebacher et al., 2014; Fox et al., 2019). Non-genetic maternal effects such as variation in egg quality (Sinervo, 1990; Bernardo, 1996; Einum and Fleming, 1999), variation in parental behavior (Champagne et al., 2003; Koch and Meunier, 2014), and traits such as maternal immune components (e.g., antibodies, Roth et al., 2018), and maternally derived hormones (Dantzer et al., 2013; Ruuskanen, 2015) have long been recognized for their potential to shape offspring phenotype and performance in response to current or expected environmental quality (Mousseau and Fox, 1998; Green, 2008). When a mother is exposed to a stressful environment during gestation or follicular recruitment, she may mount a stress response by elevating her glucocorticoid (GC) levels (Wingfield, 2013; Schreck and Tort, 2016). Recent research has examined the transfer of environmentally elevated GCs from mother to developing offspring (i.e., maternal stress) as a modulator of offspring phenotype and performance (Love et al., 2005, 2009). These GC-induced responses have been interpreted by some researchers as predictive adaptive responses in offspring expected to face with stressful environments (Gluckman et al., 2005; Marshall and Uller, 2007; Sheriff and Love, 2013). These types of adaptive response mechanisms have already been highlighted as potential drivers of flexible responses to warming environments (Meylan et al., 2012), but the role of maternal GCs as a signal of a stressor to offspring—such as warmer waters—has not been fully established. Exposure to increased maternal GCs has been shown to result in phenotypes expected to have lowered energetic demand (i.e., slower growth: Hayward and Wingfield, 2004, smaller body size: Love et al., 2005; Burton et al., 2011, and lower baseline energetics: Capelle, 2017), allowing offspring to outcompete individuals with faster growth or larger size in energetically demanding warmer waters (Messmer et al., 2017). However, this expected increase in performance of offspring exposed to increased maternal GCs within a harsher environment requires further testing (Sopinka et al., 2017). In species where there is spatial or temporal overlap in the maternal and offspring environment (Sheriff and Love, 2013), these stress-induced maternal effects may be particularly relevant for signaling offspring for stressful future environments (Capelle et al., 2017; Sopinka et al., 2017). The environmental match hypothesis suggests that when there is a match between the maternal and offspring environment (i.e., stressful maternal environment, and exposure to maternal stress via elevation in maternal GCs, respectively) the result may be higher than expected offspring performance and fitness. Although we already appreciate that elevated temperatures can lead to altered offspring phenotypes across generations (Burt et al., 2011; Jonsson and Jonsson, 2016; Le Roy et al., 2017), it is unclear whether phenotypes induced by maternal stress signals (via GCs) enable offspring to optimally respond to chronic elevated rearing temperature as well as rapid, extreme changes in water temperature, especially in at-risk species (Love et al., unpublished; Sopinka et al., 2017).

Here we examine the effects of exposure to mimicked prenatal GCs and altered rearing temperatures on the thermal performance of juvenile Chinook salmon (*Oncorhynchus tshawytscha*). Exploring whether Pacific salmon possess transgenerational stress-induced responses that may mitigate the effects of climate change is relevant from both a mechanistic and a conservation point of view. Mechanistically, Pacific

salmon are ectothermic and are highly susceptible to thermal stressors (McCullough, 1999; Geist et al., 2006; Kuehne et al., 2012; Bowerman et al., 2018). Pacific salmon have the capacity to mount a GC stress response (i.e., elevated plasma GCs) to additional environmental stressors during migration and spawning (Cook et al., 2014). Since the maternal spawning and offspring development environments overlap spatially, maternal stress has the potential to act as a reliable signal of the offspring's future environment (Healey, 1991). From a conservation standpoint, increasing river temperatures and higher frequency of droughts are predicted to highly impact cold-water species such as North American Pacific salmon (McCullough et al., 2009; Cunningham et al., 2018); multiple Pacific salmon species are in (or soon expected to be in) decline due to climate change (Crozier et al., 2008; Ford et al., 2011); and multiple populations of Chinook salmon in particular are in rapid decline (COSEWIC, 2018). Therefore, our study species and system offer an ideal combination in which to investigate environmental inducers and transgenerational signals of offspring stress. Predicting how the underlying regulatory physiology, in addition to thermotolerance capacity, will be impacted in Chinook salmon by future warming scenarios is very challenging. Recent experimental work in fish has suggested that while core physiological processes such as cardiorespiratory functions may indeed be thermally plastic, upper thermal tolerance limits may be much less flexible (Sandblom et al., 2016). Although even more difficult to predict, estimating the degree to which stress-induced transgenerational effects will further influence both of these important performance metrics remains imperative. Additional mechanistic (i.e., transcriptional profiling and physiological performance; e.g., Colson et al., 2019) studies examining the impact of thermally induced developmental plasticity in both these key systems, as well as measuring whole-animal performance under differing thermal scenarios (e.g., Farrell et al., 2008), will be required to answer these complex questions.

To examine how the effects of climate change interact with maternal GCs to generate plastic responses in Pacific salmon, we applied the environmental matching paradigm (Sheriff and Love, 2013). Specifically, we test the adaptive potential of altered offspring phenotypes (due to maternal stress and rearing temperature) to cope with environmentally relevant stressors such as increased rearing temperatures under climate change (Sheriff et al., 2017). Within this framework, we mimicked a maternal stress signal by exogenously elevating egg cortisol (via post-fertilization bathing method), and then raised resultant cortisol-dosed (and control) offspring under a current (+0°C) or an elevated (+3°C) temperature regime (**Figure 1**). At 7–9 months post fertilization we assessed the thermal performance of fish in two ways. First, we determined the CT_{Max} of the fish, defined as the temperature at which fish lose equilibrium under steadily increasing water temperatures (Chen et al., 2013; McDonnell and Chapman, 2015). This is a standardized approach in the literature to approximate thermal tolerance (Becker and Genoway, 1979; Lutterschmidt and Hutchison, 1997). Second, we quantified the energetic response of the fish following 3 days of environmentally relevant, but challenging, thermal spikes in water temperature. Given previous work that found higher rearing water temperatures lead to higher thermal tolerance (Sandblom et al., 2016), we predicted offspring raised in elevated temperatures would have a higher thermal performance (i.e., higher CT_{Max}, lower energetic cost) in both thermal performance metrics. Based on the environmental matching hypothesis, we predicted that cortisol-dosed offspring raised in elevated rearing temperatures would have a greater thermal performance than control-dosed offspring reared in the same elevated temperatures and facing the same acute thermal challenge.

MATERIALS AND METHODS

Fish Origins and Husbandry

On October 4th, 2016, we caught 15 adult female and 9 adult male spawning Chinook salmon from the Credit River, Ontario, Canada (43°34′40.0″N 79°42′06.3″W), stripped their gametes, and transferred the gametes to the University of Windsor on ice in coolers. We fertilized eggs from each female separately using pooled set of milt in 950 mL containers. We activated the sperm by using 60 mL of river water (Lehnert et al., 2018). Immediately following fertilization, we added river water mixed to 1,000 ng/mL of cortisol (H4001, Sigma-Aldrich Canada Co.) dissolved in 90% ethanol (HPLC grade, Sigma-Aldrich Canada Co.) or 0 ng/mL (ethanol and water only) to each container of eggs for our cortisol-dosed and control-dosed treatments respectively (8 containers per female: 4 cortisol-dosed and 4 control). The cortisol dose concentration was designed to increase egg cortisol levels within a biologically relevant range (within 2 SD of controls) based on previous studies (Auperin and Geslin, 2008; Sopinka et al., 2016; Capelle et al., 2017, reviewed in Sopinka et al., 2017). After a 2-h cortisol treatment, eggs were rinsed using dechlorinated water, and subset of 3 eggs per container were collected for cortisol treatment verification (See Warriner et al., 2020 for full methods and results). Eggs that were cortisol-dosed had (mean ± SD) 75.2 ± 42.4 ng/g cortisol, and control had 22.8 ± 25.4 ng/g. Each container of eggs was then further split into two cells (4-in × 3-in) within a vertical egg-incubation stack. To replicate our cortisol treatment, we placed 2 cortisol-dosed and 2 control treated containers from the same female in the same egg incubation stack (16 incubation cells per female). We then reared eggs under either current (+0°C) or elevated (+3°C) temperature regimes (one incubation stack per temperature treatment; see **Figure 1**). The current temperature regime was chosen to mimic water temperatures recorded in the Credit River through the Provincial Water Quality Monitoring Network from 2010 to 2014 (PWQMN: Ontario Ministry of Environment and Climate Change), while elevated rearing temperatures were chosen to reflect the higher end of climate change models for river temperatures (van Vliet et al., 2013; also see Zhang et al., 2018; Liu et al., 2020). This resulted in 4 treatment combinations in a 2 × 2 design: (1) current temperature reared—control, (2) current temperature reared—cortisol-dosed, (3) elevated temperature reared—control, and (4)

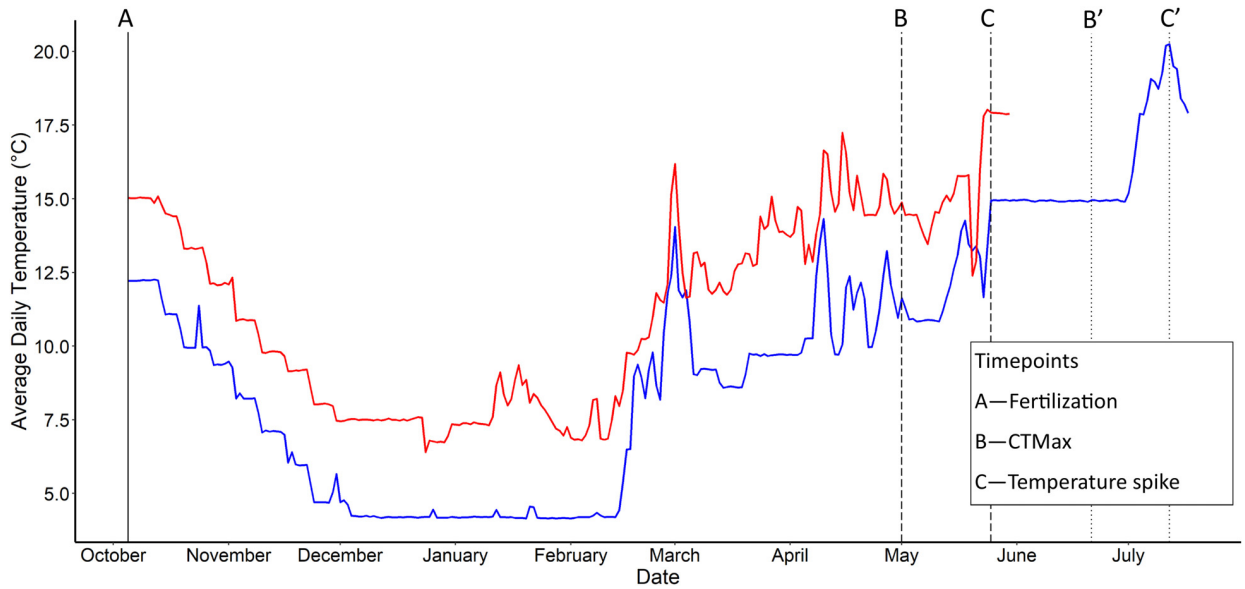

FIGURE 1 | Mean daily water temperature for the two rearing temperature regimes (in aquatic facility) examined in Chinook salmon (*Oncorhynchus tshawytscha*). Blue—current temperature regime (0°C) and red—elevated temperature regime (+3°C). Vertical lines represent sampling time points: solid—both current and elevated groups; dashed, **(B,C)**—elevated, dotted, **(B′,C′)**—current. Time points are: **(A)**—Fertilization (Oct 4th), **(B,B′)**—CTMax start date (∼14°C), and **(C,C′)**—Thermal spike start date (∼18°C). Modified from **Figure 2** found in Warriner et al. (2020); Copyright (©) 2020 Warriner, Semeniuk, Pitcher, and Love.

elevated temperature reared—cortisol-dosed. Each female's eggs were split across all four groups to account for maternal effects. All work described here was approved and completed under University of Windsor Animal Use Project Protocols (AUPPs: #14-25 and #15-15).

On Dec 23rd, 2016 and Feb 16th, 2017, for elevated- and current-reared temperature offspring respectively, we transferred fry at the exogenous-feeding stage (2–4 months post-fertilization) to 320 L recirculation-system housing tanks (five tanks per temperature treatment, with a separate system for each temperature treatment). We separated offspring by maternal identity and cortisol treatment using 10 L perforated buckets placed within the holding tanks (six buckets per tank). Buckets contained 100 offspring each (combined from replicate cortisol treatment containers and replicate incubation cells). During this period, water changes occurred at least daily to maintain water quality. The fish were housed under red light conditions following a 12:12 h light: dark cycle and were fed 3–4 times a day *ad libitum*. To ensure accurate temperature records, water temperatures in each stack were measured hourly (HOBO® Water Temperature Pro v2 Data Logger; Onset). During this period, the water temperature of the housing tanks continued to follow the current and elevated temperature seasonal regime (**Figure 1**). Due to mechanical error of an in-line chiller used to control the current-temperature treatment, housing temperatures were slowly raised with drop-in chillers to match that of the elevated-temperature treatment on March 3rd, 2017, to minimize stress. Since this overlap in temperature was for only 5 days until the chiller was repaired, and within the magnitude of temperature fluctuations found in riverine environments (Caissie, 2006), the effects of this period are expected to be minimal. Experiments were performed when fish were at similar accumulated thermal units (ATUs: **Table 1**), which has been shown to correspond with fish development (Neuheimer and Taggart, 2007).

CT_{Max}

We evaluated the acute thermal tolerance of the fish across egg cortisol and rearing temperature treatments by determining their Critical Thermal Maximum (CT_{Max}). CT_{Max} is defined as the temperature at which fish lose equilibrium (i.e., unable to maintain an upright position) under steadily increasing water temperature (Becker and Genoway, 1979; Lutterschmidt and Hutchison, 1997). These trials occurred on May 1st–7th (elevated temperature) and on June 21st–26th, 2017 (current temperature). Two experimental tanks were run concurrently, and trials ran between 08:00 and 19:00 H. Within each experimental tank, four individuals were each placed in separate tapered perforated circular buckets (top diameter 28 cm × bottom diameter 16.5 cm × deep 28 cm), with individuals per experimental tank consisting of the same maternal identity, cortisol- and rearing temperature treatment (n_{total} = 234 fish). Two air stones were used per experimental tank to ensure that dissolved oxygen levels remained high throughout the trials. These experimental tanks had the same water temperature as the housing tank at the start of the trial (± 0.7°C, starting temperature range = 13.2–15.1°C), which was controlled by an immersion circulating heater (SC100 Immersion Circulators: *Thermo Fisher Scientific*). At the end of the 1-h acclimation period, we increased temperature (by ∼0.2°C/min, $\bar{x}$ = 0.20, range = 0.13–0.35; similar to rates in Becker and Genoway, 1979) until the fish lost equilibrium. We measured water temperatures throughout the trial, and we

TABLE 1 | Starting dates for the CT_{Max} and thermal spike experiments in Chinook salmon (*Oncorhynchus tshawytscha*) with the respective accumulated thermal units (ATUs).

	CT_{Max}		Thermal spike	
	Starting date	**Accumulated thermal units (ATUs)**	**Starting date**	**Accumulated thermal units (ATUs)**
Current	21–26 June 2017	2,284–2,359	12–15 July 2017	2,637–2,694
Elevated	1–7 May 2017	2,251–2,337	25–28 June 2017	2,611–2,665

measured dissolved oxygen (DO) pre- and post-trial in a subsample of the trials ($n_{pre-trialDO}$ = 40, $\bar{x}_{pre-trialDO}$ = 8.70 mg/L, $n_{post-trialDO}$ = 37, $\bar{x}_{post-trialDO}$ = 6.83 mg/L; LabQuest 2, stainless steel temperature probe, optical DO probe: *Vernier*). Our temperature probe measured temperature to 0.1°C with ± 0.2–0.5°C accuracy at 0–100°C, respectively. The trials were filmed under red light using low-light sensitivity cameras for later video analysis. After each trial, we euthanized the eight fishes using clove oil and pithing, and measured their body mass (to 0.01 g). During video analysis, the experimenter—blind to maternal identity and cortisol treatment—recorded the time when the fish lost equilibrium for a minimum of 10 consecutive seconds, and the temperature at which this occurred.

Energetic Response to Thermal Spikes

As our second measure of thermal performance, we quantified the energetic (physiological) coping response of fish in relation to the egg cortisol and rearing temperature treatments following 3 days of thermal spikes (+9°C—one spike per day). The maximum temperature of our thermal spike was chosen from the results of our CT_{Max} experiment (slightly lower so that thermal spike was challenging but not affecting locomotion). The rate of increase in our environmentally relevant thermal spikes was chosen based on literature examining the effects of diel cycling and temperature spikes on fish energetics and metabolism (Tunnah et al., 2016; Corey et al., 2017; Gallant et al., 2017). Many of these studies have based their ramping protocols on *in situ* river temperature data. After our experiments, we were able to confirm similar spikes in temperature in the natal stream through water temperature loggers deployed in the Credit River from October 2016 to October 2017 (**Figure 2**). These trials occurred on May 25th–June 2nd and on July 12th–20th 2017 for the elevated and current treatments, respectively ($n_{replicate\ trials}$ = 4; **Figure 3**). On the evening prior to the first day of thermal spiking, 32 fish were transferred from their housing tanks into two experimental tanks. We conducted two different temperature cycle treatments: first, the spiked group which experienced three environmentally relevant thermal spikes over the 3 days, whereas the second, the constant group, was maintained at a steady temperature (∼18°C, to act as a control for potential transfer stress). In the spiked temperature treatment, thermal spikes were increased and decreased at a rate 1°C/h, resulting in a +9°C temperature spike in 18 h. Within each tank, 4 perforated buckets (top diameter 28 cm × bottom diameter 16.5 cm × deep 28 cm) each contained 3–4 randomly selected fish from the same temperature and cortisol treatment over the experimental period (n_{total} = 117). On the night of the final day, buckets were covered with opaque lids to reduce disturbance during sampling planned for the following morning. At 07:00 H of the 4th day, we removed the fish from their containers using a net, and collected their blood (within 3 min of first disturbance for each bucket) by caudal puncture using 10 μL heparinized microcapillary tubes. Each fish was then weighed (0.01 g) and placed in RNA-Later™ for a future transcriptomics project (Finerty, 2020). We transferred the blood into heparinized microcentrifuge tubes and placed these on ice, and then measured blood glucose and lactate concentrations on-site from whole blood using handheld meters within 8–13 min of whole blood being collected (Freestyle Insulinx: *Abbott Diabetes,* precision: SD ± 0.1–0.3 on range 2.4–19.2 mmol/L; Lactate Plus: *Nova Biochemical*, precision SD ± 0.06–0.49 on range 1.6–22.1 mmol/L; Barkley et al., 2016; Beecham et al., 2006; Wells and Pankhurst, 1999). After 1 h on stored on ice, the microcentrifuge tubes were centrifuged at 10,000 rpm for 12 mins, and the plasma collected and stored at −80°C for later cortisol analysis. We assayed the plasma of juvenile fish for baseline cortisol levels using non-extracted plasma and a previously validated enzyme-linked immunosorbent assay (ELISA Cortisol Kit: Cayman Chemical; Capelle, 2017). We ran samples in triplicate at a 1:50 dilution. Assay plates were read at 412 nm on a plate reader, and intra- and inter-plate variation were 2.8 and 17.5%, respectively.

Statistical Analysis

We conducted all statistical analyses in R version 3.5.1 (R Core Team, 2018). We assessed model assumptions by graphical inspection: quantile-quantile plots of the residuals to verify normality, and residuals vs. fitted values were plotted to verify homogeneity. We transformed data when assumptions were not met using a log transformation, or when needed, a Box-Cox power transformation (Osborne, 2010) in the MASS package (Venables and Ripley, 2003). We ran linear mixed models (LMM) in the lme4 package (Bates et al., 2015; see **Supplementary Table 1**).

After visually plotting CT_{Max} scores, we detected one individual score to be 3 median absolute deviations (MAD) from the median (median = 28.8°C, MAD = 0.48, datum = 26.6) and thus it was identified as a statistical outlier and removed from the dataset (Leys et al., 2013). This individual was at the smaller body mass end of our range (mass = 0.65 g), and in field notes was recorded as in poor body condition (i.e., frayed tail), which may have contributed to its earlier loss of equilibrium. For CT_{Max}, we examined the interactive effects of rearing temperature and prenatal cortisol using a model that included the fixed effects of rearing temperature × cortisol treatment interaction, and their main effects (rearing temperature + cortisol treatment) and

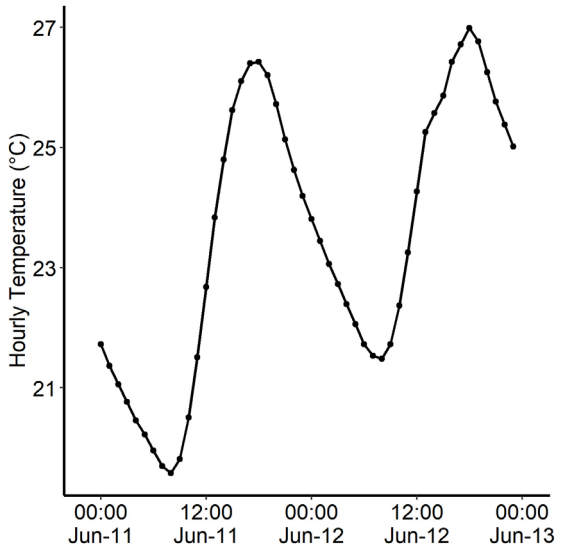

FIGURE 2 | An example of a temperature spike from the Credit River (43°34′40.″N 79°42′06.3″W) during June 11th–13th 2017 (HOBO: Water temp pro v2). Credit River was the origin population for the Chinook offspring. Highest temperature (27.0°C) reached on June 12th, 2016 at 18:00 H.

offspring body mass. We included testing tank, testing bucket (nested within tank), start temperature, and maternal identity as random effects for this model.

We analyzed the energetic response of offspring to temperature cycle (thermal spikes) using an LMM after transformation (plasma cortisol: Box-Cox, current—λ = 0.242, elevated—λ = 0.364: Osborne, 2010; glucose and lactate: log transformation). Since we *a priori* were interested in comparing energetic values within the same rearing temperature treatment, we separated the analyses into separate models for each rearing temperature. This decision also avoided the complexity of examining notoriously difficult to interpret 3-way interactions between egg cortisol treatment, water temperature treatment and the spike treatment. Within current and elevated temperature regimes, we tested models for response variables: cortisol, glucose, and lactate that included fixed effects of temperature cycle (constant or spiked), cortisol treatment, their interaction (temperature cycle × cortisol treatment), and body mass. Models also included random effects of replicate round and bucket ID.

We analyzed the interactions (CT_{Max}: rearing temperature × cortisol treatment; thermal spikes: cortisol treatment × temperature cycle) in all models by fitting them with maximum likelihood (ML) estimations, and conducting a likelihood ratio test (LRT). If the interaction was significant ($p < 0.05$), the model was refitted with restricted maximum likelihood estimation (REML), and we conducted *post-hoc* analyses using false discovery rates (FDR, sharpened method) on pairwise comparison of interest (Verhoeven et al., 2005; Pike, 2011). Using this FDR *post-hoc* approach, we report *q*-values, which are adjusted *p*-values (Pike, 2011). We calculated *q*-values, using *p*-values from the emmeans package (Lenth, 2020) and using the Excel file from Pike (2011). We calculated the difference in marginal (variance of fixed effects only) and conditional (variance of fixed and random) R^2 values of significant interactions against model without interaction (using MuMIn package; Nakagawa and Schielzeth, 2013; Barton, 2019), as a method of estimating the interaction effect size (this difference denoted by ΔR). If the interaction was instead determined to be non-significant ($p > 0.05$), it was removed from the model, and main effects were tested using LRT with ML estimations. After the final model was established for CT_{Max}, we tested the effects of maternal identity in the model (included as a random factor) using LRT. Maternal identity was not added to the statistical model of the energetic response of offspring to the temperature cycle since maternal identity could not be tracked due to experimental constraints.

RESULTS

CT_{Max}

CT_{Max} was influenced by rearing temperature, where fish that were raised in elevated temperatures had significantly higher CT_{Max} than those raised in current temperatures (χ^2 = 77.9, $p < 0.001$). There was also a marginally significant (at the 10% level—$p < 0.1$) effect of rearing temperature by cortisol treatment interaction on CT_{Max} (LMM, LRT: $\chi^2 = 2.92$, $p = 0.087$; **Figure 4** and **Table 2**). Cortisol dose alone did not significantly affect CT_{Max} ($\chi^2 = 0.13$, $p = 0.72$). Body mass had a marginally significant effect (at the 10% level) on CT_{Max} (χ^2 = 2.78, $p = 0.095$), and thus was retained within the model. In the final model, maternal identity was a significant random effect for CT_{Max} (χ^2 = 14.0, $p < 0.001$, variance = 0.018; see **Supplementary Table 2**).

Energetic Response to Thermal Spikes

Plasma Cortisol

Within the elevated temperature treatment, there was a significant cortisol treatment by temperature cycle interaction on plasma cortisol (LMM: χ^2 = 4.47, $p = 0.034$; marginal, conditional R^2 with interaction: 11.8%, 40.2%; without interaction: 0.82%, 39.7%, ΔR: 11.0%, 0.5%; **Figure 5A** and **Table 2**). Despite this overall global effect, FDR *post-hoc* analysis was unable to differentiate significant differences between all pairwise comparisons ($q \geq 0.32$), although the pattern of results suggests cortisol-exposed offspring exhibit lower plasma cortisol concentrations than control-dosed fish within the spiked temperature, and the opposite under the constant-temperature controls. Within the current water temperature treatment, the interaction of cortisol and spike treatments was non-significant (χ^2 = 1.46, $p = 0.23$), nor was the main effect of temperature cycles on plasma cortisol levels ($\chi^2 = 0.14$, $p = 0.71$). However, cortisol treatment alone was marginally significant (at 10% level: $\chi^2 = 2.90$, $p = 0.089$), where cortisol-dosed offspring had lower plasma cortisol levels than control-dosed. Body mass did not have a significant effect on plasma cortisol in both rearing temperatures (elevated: $\chi^2 = 0.01$, $p = 0.91$; current: $\chi^2 = 0.91$, $p = 0.34$).

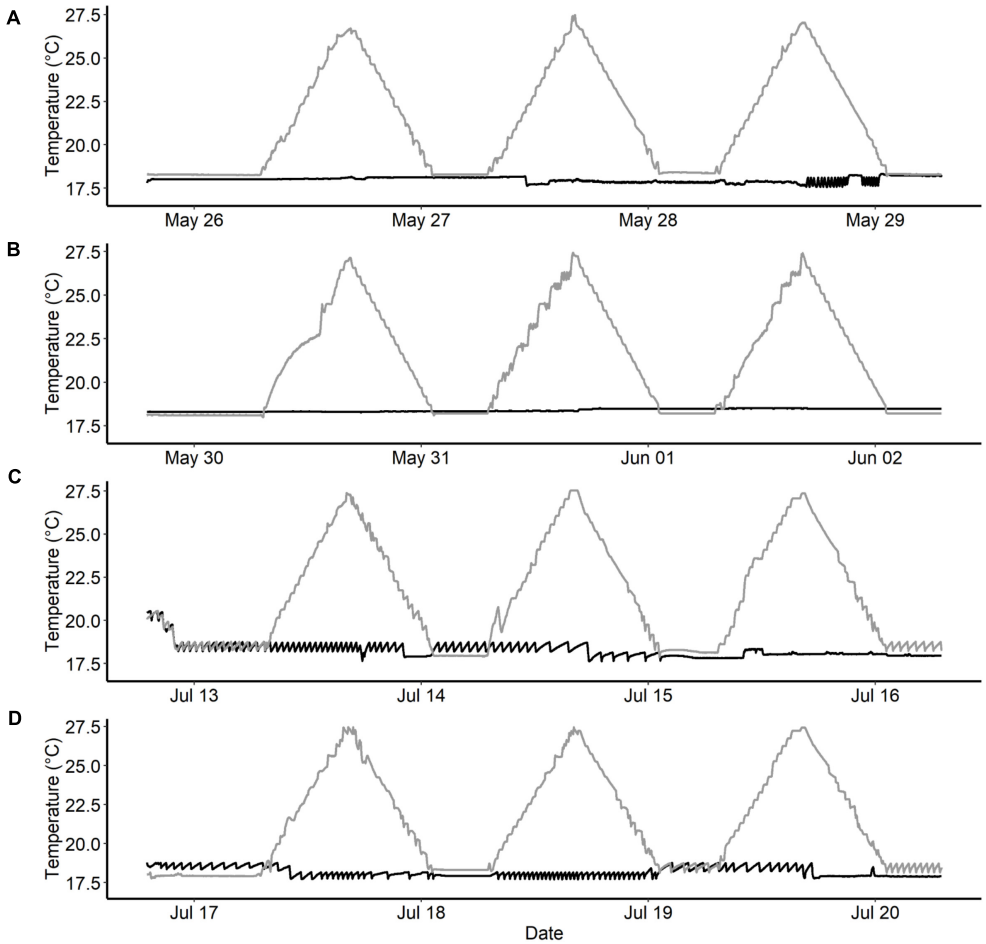

FIGURE 3 | Temperature recording from temperature loggers (HOBO: Water temp pro v2) that measured water temperature once every minute during water thermal spike studies in Chinook salmon. Black lines represent the constant temperature groups (∼18°C) and the gray line represents the spike groups (+9°C per day). **(A,B)**—represents replicate rounds for fish reared at elevated temperatures, and **(C,D)**—represents replicate rounds for fish reared at current temperatures.

Whole Blood Glucose

Within the elevated temperature treatment, there was a marginally significant effect of cortisol and temperature cycle interaction on glucose (LMM: $\chi^2 = 3.66$, $p = 0.056$; **Figure 5B**). However, when we examined cortisol and temperature cycle as main effects, they did not significantly affect blood glucose (cortisol: $\chi^2 = 2.47$, $p = 0.12$, spike: $\chi^2 = 0.025$, $p = 0.88$). Under the current temperature regime, glucose levels were not significantly impacted by cortisol and temperature cycle interaction ($\chi^2 = 0.37$, $p = 0.54$), nor cortisol treatment as a main effect ($\chi^2 = 0.15$, $p = 0.70$). However, temperature cycle as a main effect did significantly affect glucose ($\chi^2 = 4.14$, $p = 0.042$), where offspring in the spiked treatment had significantly lower glucose levels. Body mass did not have a significant effect on glucose in both rearing temperatures (elevated: $\chi^2 = 0.93$, $p = 0.34$; current: $\chi^2 = 0.06$, $p = 0.81$).

Whole Blood Lactate

Within the elevated temperature treatment, there was no cortisol by temperature cycle interaction on lactate concentrations ($\chi^2 = 0.94$, $p = 0.63$; **Figure 5C**), nor a main effect of cortisol treatment ($\chi^2 = 2.48$, $p = 0.12$). Temperature cycle had a marginally significant effect at the 10% level ($\chi^2 = 2.90$, $p = 0.09$), where fish that underwent the thermal spike treatment had marginally higher lactate levels. Overall lactate levels were also higher in fish with a larger body mass regardless of treatment ($\chi^2 = 5.68$, $p = 0.017$). Under the current rearing temperature, cortisol by temperature cycle interaction did not significantly affect lactate ($\chi^2 = 0.21$, $p = 0.65$). Lactate also did not significantly differ across cortisol treatment ($\chi^2 = 1.02$, $p = 0.31$), temperature cycle ($\chi^2 = 0.86$, $p = 0.35$), or with variation in body mass ($\chi^2 = 1.57$, $p = 0.21$).

DISCUSSION

With climate change leading to increased water temperatures and elevated daily temperature fluctuations (see Introduction), we aimed to test whether maternal stress (i.e., exogenously increased egg cortisol) mitigates the effects of chronically

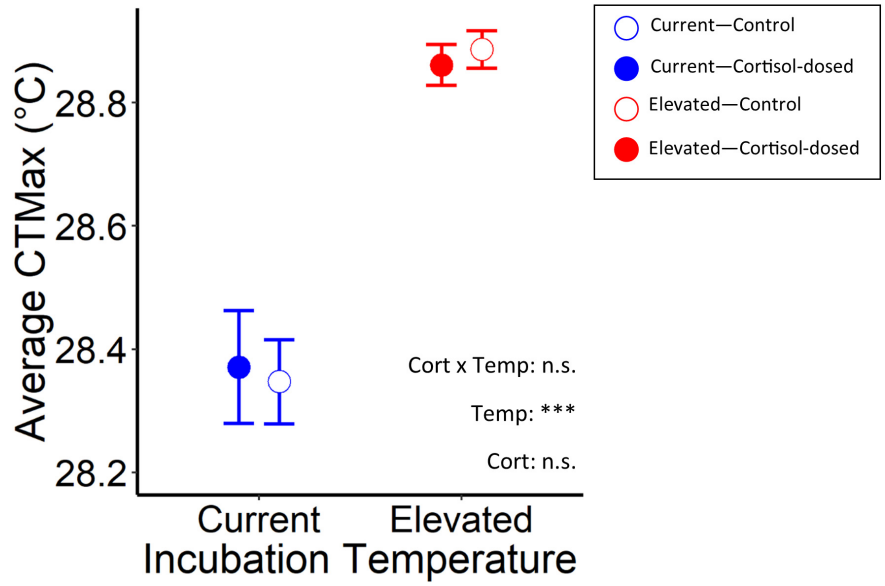

FIGURE 4 | Effects of rearing temperature and prenatal cortisol on average CT_{Max} in juvenile Chinook salmon exposed to control (open circles) and cortisol-exposed (closed circles) treatments and raised in current (blue), or elevated (red) temperature regimes (n.s. and *** represent *p*-values that were >0.05 and <0.001, respectively).

TABLE 2 | Thermal tolerance performance and phenotype metrics (mean ± SE) of juvenile Chinook salmon.

		Temperature cycle	Current cortisol-dosed	N	Current control	N	Elevated cortisol-dosed	N	Elevated control	N
CT_{Max}	CT_{Max} (°C)	–	28.4 ± 0.09	59	28.3 ± 0.07	57	28.9 ± 0.03	59	28.9 ± 0.03	58
	Mass (g)	–	1.77 ± 0.14	59	1.74 ± 0.13	57	2.20 ± 0.15	59	2.14 ± 0.14	58
Temperature cycle	Cortisol (ng/mL)	Constant	24.4 ± 5.8	15	55.8 ± 9.8	14	58.1 ± 12.4	14	30.3 ± 6.0	15
		Spike	43.3 ± 9.8	15	42.5 ± 5.9	15	21.8 ± 3.4	15	50.2 ± 8.2	14
	Glucose (mmol/l)	Constant	2.29 ± 0.08	15	2.39 ± 0.11	14	2.53 ± 0.15	14	2.16 ± 0.09	15
		Spike	2.17 ± 0.09	15	2.16 ± 0.09	15	2.31 ± 0.05	15	2.32 ± 0.09	14
	Lactate (mmol/l)	Constant	2.03 ± 0.08	15	2.29 ± 0.18	14	2.10 ± 0.18	14	1.73 ± 0.12	15
		Spike	2.30 ± 0.18	15	2.48 ± 0.27	15	2.23 ± 0.17	15	2.00 ± 0.21	14
	Mass (g)	Constant	4.45 ± 0.53	15	4.40 ± 0.40	14	5.94 ± 0.72	14	4.62 ± 0.60	15
		Spike	4.00 ± 0.34	15	4.48 ± 0.32	15	4.64 ± 0.62	15	3.57 ± 0.55	14

elevated rearing temperatures to enhance offspring responses to extreme temperature variation (i.e., spikes) in juvenile salmon. Under the environmental match hypothesis (Love et al., 2013; Sheriff and Love, 2013), when offspring were reared under elevated water temperatures (stressful environment) we predicted that exposure to elevated egg cortisol (resulting from mimicking maternal stress) would improve thermal performance (environmental match) compared to control-dosed individuals (mismatch). Similar to established work on acclimation temperature (McDonnell and Chapman, 2015), we demonstrated that elevated rearing temperatures can indeed enhance offspring tolerance to rapid increases in temperature (i.e., CT_{Max}). Contrary to our predictions, mimicking a signal of maternal stress (exogenous increase in egg cortisol) did not noticeably modulate offspring thermal sensitivity (both CT_{Max} and energetic response) within either rearing temperature regime, with the exception of marginally lower plasma cortisol in cortisol-dosed offspring reared under current temperatures.

CT_{Max}

Although rearing temperature impacted offspring maximum thermal tolerance, thermal tolerance was not further altered by exposure to elevated egg cortisol: fish raised in elevated temperatures had a higher mean CT_{Max} than fish reared in current temperatures. Previous work has shown that short-term acclimation to higher temperatures results in a higher CT_{Max} (Zhang and Kieffer, 2014; McDonnell and Chapman, 2015). Long-term rearing (from early development) in elevated temperatures also led to increased CT_{Max} (He et al., 2014; Muñoz et al., 2017; Del Rio et al., 2019; but see Chen et al., 2013). Thus, acclimation to higher temperatures may allow for organisms to persist within a warmer world under climate change. However, acclimation to higher rearing temperature

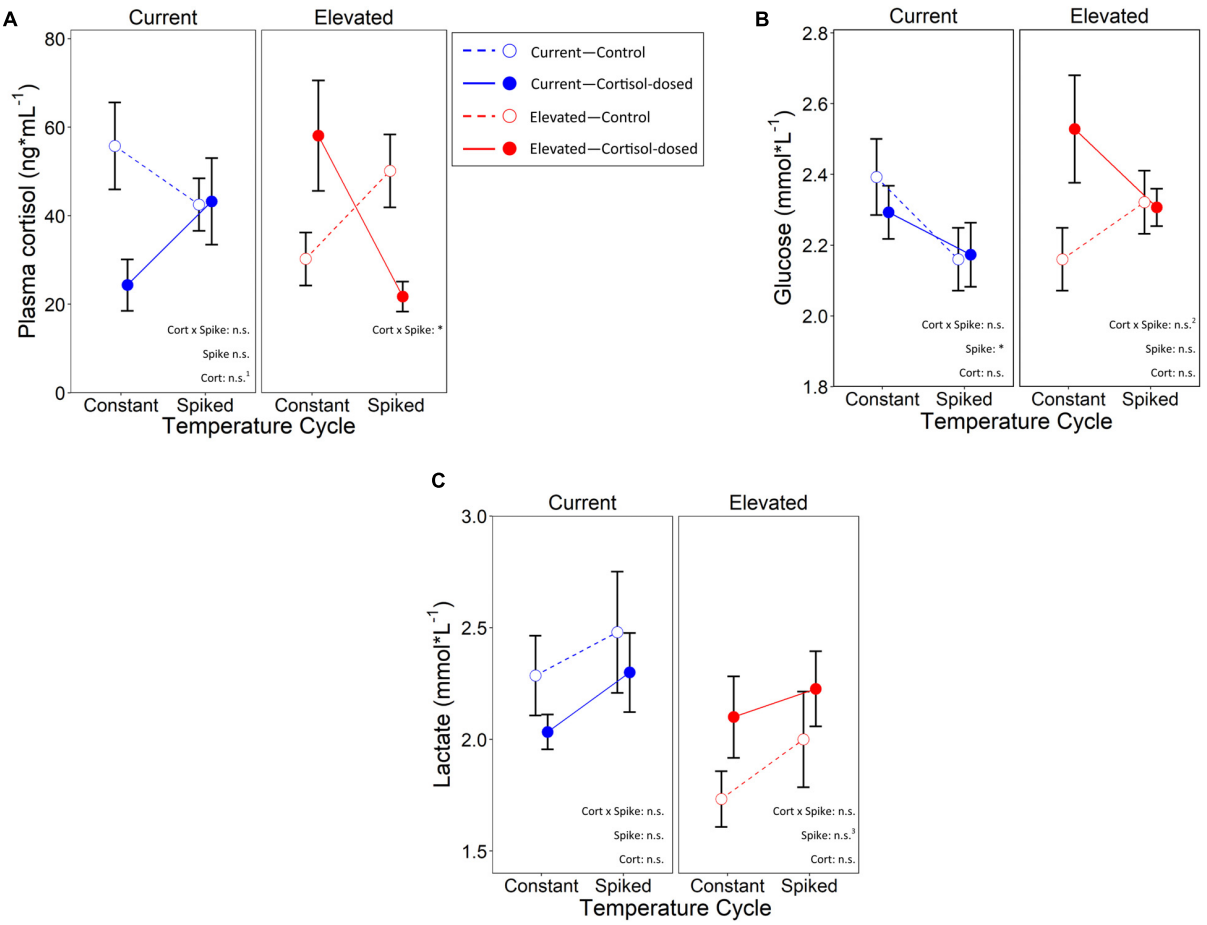

FIGURE 5 | Effects of cortisol treatment and temperature cycle on **(A)**—plasma cortisol, **(B)**—whole blood glucose, and **(C)**—whole blood lactate in juvenile Chinook salmon. Open circles depict control and closed circles depict cortisol-dosed. For the temperature cycle groups, constant indicates fish remained at a constant temperature, and spiked are fish who were exposed to 3 days of thermal spikes. n.s. and *represent a p-value of >0.05 and <0.05, respectively. n.s.[1], n.s.[2], and n.s.[3] represents a marginal significance of $p = 0.089$, $p = 0.056$, and $p = 0.090$ respectively.

does not increase CT_{Max} in a 1:1 ratio (i.e., a ceiling threshold exists; Sandblom et al., 2016), thus acclimation to higher temperatures may have a limited capacity to increase thermal tolerance. Unlike elevated rearing temperatures, exposure to elevated egg cortisol did not further augment juvenile salmon CT_{Max}. To our knowledge, this is the first study to test the effects of prenatal stress (i.e., exogenously elevated egg cortisol) on CT_{Max}. Previous studies have proposed that intergenerational or transgenerational effects may be a mechanism by which fish may increase their thermal tolerance under climate change (Munday, 2014), although exposure to exogenously elevated prenatal cortisol does not appear to be a significant maternal effect contributor to thermal tolerance and performance. Overall, we also found that CT_{Max} was shaped by variation in juvenile body mass, where larger individuals had a higher CT_{Max}. Mass has previously been shown to alter CT_{Max}, with some studies reporting that larger fish (higher mass, length, or body condition) have higher CT_{Max}, which is thought to be driven by higher energetic reserves (Chen et al., 2013; Gallant et al., 2017). While others have found the opposite, with smaller fish having higher CT_{Max}, potentially due to the have lower oxygen limited energetic demand of smaller fish (Di Santo and Lobel, 2017; Messmer et al., 2017). Although not the main aim of our study, we also found maternal identity to be highly influential on offspring CT_{Max}. Maternal effects are thought to play a significant role in determining how organisms respond to elevated temperatures (Burt et al., 2011), and our study provides further evidence that the role of maternal identity on offspring phenotype should be further studied.

Energetic Coping to Thermal Spikes

Overall, our study suggests that exposure to elevated egg cortisol may decrease plasma cortisol responses during thermal spikes for fish raised in elevated temperatures; however, these pair-wise, independent effects require further investigation as they were not detectable in *post-hoc* analysis. By rearing these fish under elevated temperatures, the offspring may be more prepared for thermal spikes, which is why we may not have seen an impact of thermal spikes on plasma cortisol. However, within the current (i.e., benign) temperature treatment, cortisol-dosed offspring

had marginally lower plasma cortisol regardless of whether the offspring had undergone the thermal spike treatment. According to the environmental match hypothesis, the cortisol-exposed offspring should be mismatched to the current rearing environment, and yet, they had significantly lower baseline plasma cortisol (i.e., a lower energetic demand) than control fish. Previous work has shown that cortisol-dosed salmonid offspring had a lower plasma cortisol in a semi-natural postnatal environment (compared to low water conditions: Capelle, 2017) and in laboratory conditions compared to controls (Colson et al., 2015). However, for each group in the thermal spikes experiment, the average plasma cortisol was >25 ng/ml, with the exception of cortisol-dosed fish in the constant group reared at current temperatures (24.4 ng/mL). Compared to previous studies in juvenile Chinook salmon (Capelle, 2017; Dender et al., 2018), the combined average for cortisol-dosed offspring (33.9 ng/mL) is within the biologically relevant range for this species. This suggests that the lower plasma cortisol responses we report for the cortisol-dosed fish may be an adaptive energy-saving response compared to control offspring, which in turn may have long-term phenotypic consequences such as higher growth (Wendelaar-Bonga, 1997).

Among offspring reared under elevated temperature, there was a marginally significant ($p < 0.06$) interaction between cortisol dose and temperature cycle on blood glucose, suggesting cortisol-dosed offspring had higher blood glucose than controls when kept at constant temperatures. It is somewhat difficult to determine the origin of this response since having a higher glucose level may be indicative of both glucose mobilization as part of a stress response, or higher food intake (since fish were fed throughout the experiment; Polakof et al., 2012). Previous studies have found that fish cease feeding in warmer temperatures (Breau et al., 2011), and thus continued feeding may indicate that cortisol-dosed fish are retaining the ability to maintain homeostasis after exposure to a chronic thermal stressor. Regardless, the temperature cycle treatment itself did not impact blood glucose levels, suggesting that living in chronically warmer waters may allow fish to recover more quickly (i.e., via acclimation) to thermal spikes and maintain higher blood glucose levels. In support of this, Barton et al. (1987) found that rainbow trout (*Oncorhynchus mykiss*) exposed to 10 weeks of daily handling stress acclimated to additional handling and did not increase their glucose levels as a result. Similarly, a study comparing energetic responses to exposure between one and multiple thermal spikes found that after multiple thermal spikes, fish were able to maintain glucose levels by inducing anabolic metabolism and replenishing glycogen reserves (Callaghan et al., 2016). Since elevated-temperature reared fish were chronically exposed to increased temperatures, they may be using an anabolic phenotype to respond to thermal spikes, potentially allowing for the maintenance of higher glucose levels. Alternatively, when reared under current temperatures, fish undergoing thermal spikes had lower glucose levels than fish under stable temperatures, regardless of egg cortisol treatment. This suggests that fish undergoing the thermal spikes may have incurred an energetic cost, thus reducing their reserves and glucose homeostatic concentrations when raised in a benign environment.

Across both rearing temperatures, neither cortisol treatment nor temperature cycle influenced offspring whole blood lactate, although among fish reared in elevated temperatures, blood lactate was higher in fish with a larger body size. Since we ensured that oxygen levels remained high during the thermal spikes (via air stones), anaerobic metabolism may not have been necessary for the fish to persist at the higher, spiking temperatures, which is why we did not detect differences across temperature cycle treatments. Furthermore, since lactate is a by-product of anaerobic metabolism in muscle tissues (Dando, 1969), larger fish having higher lactate levels may be due to a higher proportion of muscle tissue available.

Potential for Maternal Stress to Adjust Offspring Thermal Performance

Overall, we did not find that a biologically relevant exogenous elevation of egg cortisol noticeably improved thermal tolerance in fish already raised under elevated water temperatures. Contrary to predictions of the environmental match hypothesis, elevated egg cortisol did not affect CT_{Max}, nor did this treatment consistently interact with rearing temperature to clearly modulate the suite of energetic response in fish to thermal spikes. We did find that exposure to elevated egg cortisol led to increased blood glucose in offspring reared under elevated water temperatures, suggesting these offspring have more energy readily available to cope with additional thermal stressors within elevated temperatures. Maternal GCs are thought to enact phenotypic changes in offspring via GC-glucocorticoid receptor complex induced transcription and/or epigenetic programming (Love et al., 2013; Sopinka et al., 2017). Although prenatal stress has been shown to improve energetic responses to stressful postnatal environments (e.g., low water conditions: Capelle, 2017), and has been shown to alter a host of offspring traits (Sloman, 2010; Burton et al., 2011; Sopinka et al., 2017), our study suggests that maternally derived GCs may not noticeably improve thermal tolerance under elevated water temperature conditions in Chinook salmon. Thus, maternal GCs transferred to the egg may not be able to adjust the thermal tolerances of developing offspring via environmental matching, especially in combination with a powerful modulator of offspring development such as elevated rearing temperatures. However, we examined the effect of only one cortisol dose, which may have not affected offspring thermal tolerance because the chosen cortisol dose may not have matched the intensity of the chronically elevated rearing temperatures. To determine if a prenatal cortisol signal could improve offspring thermal tolerance under elevated temperatures, more research using a variety of prenatal cortisol doses is needed. By designing experiments using only mechanisms of transgenerational effects (such as maternal GCs, epigenetics, and maternal provisioning), researchers can differentiate between the paradigms of predictive adaptive responses and the silver spoon hypothesis (Engqvist and Reinhold, 2016). Other mechanisms of transgenerational effects

(parental cues such as epigenetics and maternal provisioning) should be studied as they may allow for a mechanism by which environmental matching can improve offspring performance in warming waters (Munday, 2014; Donelan et al., 2020). In experiments when both parents and offspring are exposed to elevated temperatures, there is evidence for environmental matching which results in improved thermal tolerance in offspring (Sandblom et al., 2016; Le Roy et al., 2017; Le Roy and Seebacher, 2018). For example, Donelson et al. (2012) found that fish reared under elevated water temperatures were able to maintain their aerobic scope (an indicator of ability to do aerobic activities) if their parents were also raised in elevated temperatures. However, meta-analyses of transgenerational effects have shown mixed evidence for environmental matching inducing predictive adaptive responses (Uller et al., 2013; Yin et al., 2019). Thus, further testing the role of transgenerational stress within the environmental context of climate change remains imperative to determine the precise role of mechanisms such as maternally derived hormones (Meylan et al., 2012), and epigenetics (Anastasiadi et al., 2017; Ryu et al., 2018) in mediating adaptive responses to global warming. Future work should include examining which potential mechanisms of transgenerational effects may allow for environmental matching to improve offspring thermal tolerance by testing the effects of elevated temperatures on these parental cues. From an evolutionary perspective, our results show that offspring performance responses may be modulated by environmentally and ecologically relevant stressors (i.e., elevated rearing temperatures) experienced in early life. From an applied point of view, this project demonstrates that developmental plasticity (via early life environmental cues) may enable adaptive organismal responses to the effects of climate change.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care Committee, University of Windsor AUPP #14-25, #15-15.

AUTHOR CONTRIBUTIONS

TW, CS, and OL collected the data. TW, CS, and OL ran statistical analysis and led writing of the manuscript with input from all authors. All authors contributed to the concept and design of the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.548939/full#supplementary-material

REFERENCES

Alcorn, S. W., Murray, A. L., and Pascho, R. J. (2002). Effects of rearing temperature on immune functions in sockeye salmon (*Oncorhynchus nerka*). *Fish Shellfish Immun.* 12, 303–334. doi: 10.1006/fsim.2001.0373

Anastasiadi, D., Díaz, N., and Piferrer, F. (2017). Small ocean temperature increases elicit stage-dependent changes in DNA methylation and gene expression in a fish, the European sea bass. *Sci. Rep.* 7, 1–12. doi: 10.1038/s41598-017-10861-6

Auperin, B., and Geslin, M. (2008). Plasma cortisol response to stress in juvenile rainbow trout is influenced by their life history during early development and by egg cortisol content. *Gen. Comp. Endocrinol.* 158, 234–239. doi: 10.1016/j.ygcen.2008.07.002

Barkley, A. N., Cooke, S. J., Fisk, A. T., Hedges, K., and Hussey, N. E. (2016). Capture-induced stress in deep-water Arctic fish species. *Polar Biol.* 40, 213–220. doi: 10.1007/s00300-016-1928-8

Barton, B., Schreck, C., and Barton, L. (1987). Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. *Dis. Aquat. Organ.* 2, 173–185. doi: 10.3354/dao002173

Barton, K. (2019). *MuMIn:Multi-model inference. R package version 1.43.15.*

Bates, D., Mächler, M., Bolker, B. M., and Walker, S. C. (2015). Fitting linear mixed-effects using lme4. *Psychol. Med.* 45, 361–373. doi: 10.1017/S0033291714001470

Becker, C. D., and Genoway, R. G. (1979). Evaluation of the critical thermal maximum for determining thermal tolerance of freshwater fish. *Env. Biol. Fish* 4, 245–256. doi: 10.1007/BF00005481

Beecham, R. V., Small, B. C., and Minchew, C. D. (2006). Using portable lactate and glucose meters for catfish research: acceptable alternatives to established laboratory methods? *N. Am. J. Aqu.* 68, 291–295. doi: 10.1577/A05-074.1

Bernardo, J. (1996). The particular maternal effect of propagule size, especially egg size: patterns, models, quality of evidence and interpretation. *Am. Zool.* 36, 216–236. doi: 10.1093/icb/36.2.216

Bickford, D., Howard, S. D., Ng, D. J. J., and Sheridan, J. A. (2010). Impacts of climate change on the amphibians and reptiles of Southeast Asia. *Biodivers. Conserv.* 19, 1043–1062. doi: 10.1007/s10531-010-9782-4

Bowerman, T., Roumasset, A., Keefer, M. L., Sharpe, C. S., and Caudill, C. C. (2018). Prespawn mortality of female chinook salmon increases with water temperature and percent hatchery origin. *Trans. Am. Fish. Soc.* 147, 31–42. doi: 10.1002/tafs.10022

Breau, C., Cunjak, R. A., and Peake, S. J. (2011). Behaviour during elevated water temperatures: can physiology explain movement of juvenile Atlantic salmon to cool water? *J. Anim. Ecol.* 80, 844–853. doi: 10.1111/j.1365-2656.2011.01828.x

Buckley, L. B., and Huey, R. B. (2016). How extreme temperatures impact organisms and the evolution of their thermal tolerance. *Integr. Comp. Biol.* 56, 98–109. doi: 10.1093/icb/icw004

Burt, J. M., Hinch, S. G., and Patterson, D. A. (2011). The importance of parentage in assessing temperature effects on fish early life history: a review of the experimental literature. *Rev. Fish Biol. Fish.* 21, 377–406. doi: 10.1007/s11160-010-9179-1

Burton, T., Hoogenboom, M. O., Armstrong, J. D., Groothuis, T. G. G., and Metcalfe, N. B. (2011). Egg hormones in a highly fecund vertebrate: do they influence offspring social structure in competitive conditions? *Funct. Ecol.* 25, 1379–1388. doi: 10.1111/j.1365-2435.2011.01897.x

Caissie, D. (2006). The thermal regime of rivers: a review. *Freshw. Biol.* 51, 1389–1406. doi: 10.1111/j.1365-2427.2006.01597.x

Callaghan, N. I., Tunnah, L., Currie, S., and MacCormack, T. J. (2016). Metabolic adjustments to short-term diurnal temperature fluctuation in the rainbow Trout (*Oncorhynchus mykiss*). *Physiol. Biochem. Zool.* 89, 498–510. doi: 10.1086/688680

Capelle, P. M. (2017). *Interactive Effects of Pre and Post Natal Stressors on Chinook Salmon Performance and Fitness*. Master Thesis, University of Windsor, Windsor, ON.

Capelle, P. M., Semeniuk, C. A. D., Sopinka, N. M., Heath, J. W., and Love, O. P. (2017). Prenatal stress exposure generates higher early survival and smaller size without impacting developmental rate in a Pacific salmon. *J. Exp. Zool. Part A Ecol. Genet. Physiol.* 325, 641–650. doi: 10.1002/jez.2058

Carroll, S. P., Hendry, A. P., Reznick, D. N., and Fox, C. W. (2007). Evolution on ecological time-scales. *Funct. Ecol.* 21, 387–393. doi: 10.1111/j.1365-2435.2007.01289.x

Champagne, F. A., Francis, D. D., Mar, A., and Meaney, M. J. (2003). Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiol. Behav.* 79, 359–371. doi: 10.1016/S0031-9384(03)00149-5

Chen, Z., Anttila, K., Wu, J., Whitney, C., Hinch, S., and Farrell, A. (2013). Optimum and maximum temperature of sockeye salmon (*Oncorhynchus nerka*) populations hatched at different temperatures. *Can. J. Zool.* 91, 265–274. doi: 10.1139/cjz-2012-0300

Chevin, L. M., Lande, R., and Mace, G. M. (2010). Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. *PLoS Biol.* 8:e1000357. doi: 10.1371/journal.pbio.1000357

Cingi, S., Keinänen, M., and Vuorinen, P. J. (2010). Elevated water temperature impairs fertilization and embryonic development of whitefish *Coregonus lavaretus*. *J. Fish Biol.* 76, 502–521. doi: 10.1111/j.1095-8649.2009.02502.x

Clarke, A., and Johnston, N. M. (1999). Scaling of metabolic rate with body mass and temperature in teleost fish. *J. Anim. Ecol.* 68, 893–905. doi: 10.1046/j.1365-2656.1999.00337.x

Colson, V., Nguyen, T., Cousture, M., Damasceno, D., Le Cam, A., Bobe, J., et al. (2019). Maternal temperature exposure impairs emotional and cognitive responses and triggers dysregulation of neurodevelopment genes in fish. *PeerJ* 7:e6338. doi: 10.7717/peerj.6338

Colson, V., Valotaire, C., Geffroy, B., and Kiilerich, P. (2015). Egg cortisol exposure enhances fearfulness in larvae and juvenile rainbow trout. *Ethology* 121, 1–11.

Cook, K. V., Crossin, G. T., Patterson, D. A., Hinch, S. G., Gilmour, K. M., and Cooke, S. J. (2014). The stress response predicts migration failure but not migration rate in a semelparous fish. *Gen. Comp. Endocrinol.* 202, 44–49. doi: 10.1016/j.ygcen.2014.04.008

Corey, E., Linnansaari, T., Cunjak, R. A., and Currie, S. (2017). Physiological effects of environmentally relevant, multi-day thermal stress on wild juvenile Atlantic salmon (*Salmo salar*). *Conserv. Physiol.* 5:cox014. doi: 10.1093/conphys/cox014

COSEWIC (2018). *COSEWIC Wildlife Species Assessments, November 2018*. Available online at: https://www.canada.ca/content/dam/eccc/documents/pdf/cosewic/wsam-results/november-2018/2018-wildlife-species-assessments-detailed-november-en.pdf (accessed March 4, 2019).

Crozier, L. G., Hendry, A. P., Lawson, P. W., Quinn, T. P., Mantua, N. J., Battin, J., et al. (2008). Potential responses to climate change in organisms with complex life histories: evolution and plasticity in Pacific salmon. *Evol. Appl.* 1, 252–270. doi: 10.1111/j.1752-4571.2008.00033.x

Cunningham, C. J., Westley, P. A. H., and Adkison, M. D. (2018). Signals of large scale climate drivers, hatchery enhancement, and marine factors in Yukon River Chinook salmon survival revealed with a Bayesian life history model. *Glob. Chang. Biol.* 24, 4399–4416. doi: 10.1111/gcb.14315

Dando, P. R. (1969). Lactate metabolism in fish. *J. Mar. Biol. Assoc. U.K.* 49, 209–223. doi: 10.1017/S002531540004652X

Dantzer, B., Newman, A. E. M., Boonstra, R., Palme, R., Boutin, S., Humphries, M. M., et al. (2013). Density triggers maternal hormones that increase adaptive offspring growth in a wild mammal. *Science* 1215, 1215–1218. doi: 10.1126/science.1235765

Del Rio, A. M., Davis, B. E., Fangue, N. A., and Todgham, A. E. (2019). Combined effects of warming and hypoxia on early life stage Chinook salmon physiology and development. *Conserv. Physiol.* 7, 1–14. doi: 10.1093/conphys/coy078

Dender, M. G. E., Capelle, P. M., Love, O. P., Heath, D. D., Heath, J. W., and Semeniuk, C. A. D. (2018). Phenotypic integration of behavioural and physiological traits is related to variation in growth among stocks of Chinook salmon. *Can. J. Fish. Aquat. Sci.* 75, 2271–2279. doi: 10.1139/cjfas-2017-0367

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., et al. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci. U.S.A.* 105, 6668–6672. doi: 10.1073/pnas.0709472105

Di Santo, V., and Lobel, P. S. (2017). Body size and thermal tolerance in tropical gobies. *J. Exp. Mar. Bio. Ecol.* 487, 11–17. doi: 10.1016/j.jembe.2016.11.007

Dillon, M. E., Wang, G., and Huey, R. B. (2010). Global metabolic impacts of recent climate warming. *Nature* 467, 704–706. doi: 10.1038/nature09407

Donelan, S. C., Hellmann, J. K., Bell, A. M., Luttbeg, B., Orrock, J. L., Sheriff, M. J., et al. (2020). Transgenerational plasticity in human-altered environments. *Trends Ecol. Evol.* 35, 115–124. doi: 10.1016/j.tree.2019.09.003

Donelson, J. M., Munday, P. L., McCormick, M. I., and Pitcher, C. R. (2012). Rapid transgenerational acclimation of a tropical reef fish to climate change. *Nat. Clim. Chang.* 2, 30–32. doi: 10.1038/nclimate1323

Einum, S., and Fleming, I. A. (1999). Maternal effects of egg size in brown trout (*Salmo trutta*): norms of reaction to environmental quality. *Proc. R. Soc. B Biol. Sci.* 266, 2095–2100. doi: 10.1098/rspb.1999.0893

Enders, E. C., and Boisclair, D. (2016). Effects of environmental fluctuations on fish metabolism: atlantic salmon Salmo salar as a case study. *J. Fish Biol.* 88, 344–358. doi: 10.1111/jfb.12786

Engqvist, L., and Reinhold, K. (2016). Adaptive trans-generational phenotypic plasticity and the lack of an experimental control in reciprocal match/mismatch experiments. *Methods Ecol. Evol.* 7, 1482–1488. doi: 10.1111/2041-210X.12618

Farrell, A. P., Hinch, S. G., Cooke, S. J., Patterson, D. A., Crossin, G. T., Lapointe, M., et al. (2008). Pacific salmon in hot water: applying aerobic scope models and biotelemetry to predict the success of spawning migrations. *Physiol. Biochem. Zool.* 81, 697–708. doi: 10.1086/592057

Finerty, C. J. (2020). *A Transcriptomic Approach to Examining the Effects of Prenatal and Thermal Stress on Developmental Plasticity in Chinook Salmon (Oncorhynchus tshawytscha)*. Master's thesis, University of Windsor, Windsor, ON.

Ford, M., Cooney, T., Mcelhany, P., Sands, N., Weitkamp, L., Hard, J., et al. (2011). *Status Review Update for Pacific Salmon and Steelhead Listed Under the Endangered Species Act*. NMFS-NWFSC-113. Washington, DC: U.S. Department of Commerce.

Forsman, A. (2015). Rethinking phenotypic plasticity and its consequences for individuals, populations and species. *Heredity* 115, 276–284. doi: 10.1038/hdy.2014.92

Fox, R. J., Donelson, J. M., Schunter, C., Ravasi, T., and Gaitán-Espitia, J. D. (2019). Beyond buying time: the role of plasticity in phenotypic adaptation to

rapid environmental change. *Philos. Trans. R. Soc. B Biol. Sci.* 374:20180174. doi: 10.1098/rstb.2018.0174

Franklin, C. E., Davison, W., and Seebacher, F. (2007). Antarctic fish can compensate for rising temperatures: thermal acclimation of cardiac performance in Pagothenia borchgrevinki. *J. Exp. Biol.* 210, 3068–3074. doi: 10.1242/jeb.003137

Gallant, M. J., Leblanc, S., Maccormack, T. J., and Currie, S. (2017). Physiological responses to a short-term, environmentally realistic, acute heat stress in Atlantic salmon, *Salmo salar*. *Facets* 2, 330–341. doi: 10.1139/facets-2016-0053

Geist, D. R., Abernethy, C. S., Hand, K. D., Cullinan, V. I., Chandler, J. A., and Groves, P. A. (2006). Survival, development, and growth of Fall Chinook salmon embryos, alevins, and fry exposed to variable thermal and dissolved oxygen regimes. *Trans. Am. Fish. Soc.* 135, 1462–1477. doi: 10.1577/T05-294.1

Gluckman, P. D., Hanson, M. A., and Spencer, H. G. (2005). Predictive adaptive responses and human evolution. *Trends Ecol. Evol.* 20, 527–533. doi: 10.1016/j.tree.2005.08.001

Green, B. S. (2008). "Maternal effects in fish populations," in *Advances in Marine Bology*, ed. D. W. Sims (Cambridge, MA: Academic press), 1–105. doi: 10.1016/s0065-2881(08)00001-1

Hayward, L. S., and Wingfield, J. C. (2004). Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. *Gen. Comp. Endocrinol.* 135, 365–371. doi: 10.1016/j.ygcen.2003.11.002

He, Y., Wu, X., Zhu, Y., Li, H., Li, X., and Yang, D. (2014). Effect of rearing temperature on growth and thermal tolerance of Schizothorax (Racoma) kozlovi larvae and juveniles. *J. Therm. Biol.* 46, 24–30. doi: 10.1016/j.jtherbio.2014.09.009

Healey, M. C. (1991). "Life history of Chinook Salmon (Oncorhynchus tshawytscha)," in Pacific Salmon Life Histories, eds C. Groot and L. Margolis (Vancouver: University of British Columbia Press).

Hendry, A. P., Farrugia, T. J., and Kinnison, M. T. (2008). Human influences on rates of phenotypic change in wild animal populations. *Mol. Ecol.* 17, 20–29. doi: 10.1111/j.1365-294X.2007.03428.x

Jonsson, B., and Jonsson, N. (2016). Trans-generational maternal effect: temperature influences egg size of the offspring in Atlantic salmon *Salmo salar*. *J. Fish Biol.* 89, 1482–1487. doi: 10.1111/jfb.13040

Koch, L. K., and Meunier, J. (2014). Mother and offspring fitness in an insect with maternal care: phenotypic trade-offs between egg number, egg mass and egg care. *BMC Evol. Biol.* 14:125. doi: 10.1186/1471-2148-14-125

Kuehne, L. M., Olden, J. D., and Duda, J. J. (2012). Cost of living for juvenile Chinook salmon (Oncorhynchus tshawytscha) in an increasing warming and invaded world. *Can. J. Fish. Aquat. Sci.* 69, 1621–1630. doi: 10.1139/f2012-094

Le Roy, A., Loughland, I., and Seebacher, F. (2017). Differential effects of developmental thermal plasticity across three generations of guppies (*Poecilia reticulata*): canalization and anticipatory matching. *Sci. Rep.* 7, 1–12. doi: 10.1038/s41598-017-03300-z

Le Roy, A., and Seebacher, F. (2018). Transgenerational effects and acclimation affect dispersal in guppies. *Funct. Ecol.* 32, 1819–1831. doi: 10.1111/1365-2435.13105

Lehnert, S. J., Helou, L., Pitcher, T. E., Heath, J. W., and Heath, D. D. (2018). Sperm competition, but not major histocompatibility divergence, drives differential fertilization success between alternative reproductive tactics in Chinook salmon. *J. Evol. Biol.* 31, 88–97. doi: 10.1111/jeb.13199

Lenth, R. (2020). *emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.4.7*.

Leys, C., Ley, C., Klein, O., Bernard, P., and Licata, L. (2013). Detecting outliers: do not use standard deviation around the mean, use absolute deviation around the median. *J. Exp. Soc. Psychol.* 49, 764–766. doi: 10.1016/j.jesp.2013.03.013

Lind, M. I., and Spagopoulou, F. (2018). Evolutionary consequences of epigenetic inheritance. *Heredity* 121, 205–209. doi: 10.1038/s41437-018-0113-y

Liu, S., Xie, Z., Liu, B., Wang, Y., Gao, J., Zeng, Y., et al. (2020). Global river water warming due to climate change and anthropogenic heat emission. *Glob. Planet. Change* 193:103289. doi: 10.1016/j.gloplacha.2020.103289

Love, O. P., Chin, E. H., Wynne-Edwards, K. E., and Williams, T. D. (2005). Stress hormones: a link between maternal condition and sex-biased reproductive investment. *Am. Nat.* 166, 751–766. doi: 10.1086/497440

Love, O. P., Gilchrist, H. G., Bêty, J., Wynne-Edwards, K. E., Berzins, L., and Williams, T. D. (2009). Using life-histories to predict and interpret variability in yolk hormones. *Gen. Comp. Endocrinol.* 163, 169–174. doi: 10.1016/j.ygcen.2008.10.001

Love, O. P., McGowan, P. O., and Sheriff, M. J. (2013). Maternal adversity and ecological stressors in natural populations: the role of stress axis programming in individuals, with implications for populations and communities. *Funct. Ecol.* 27, 81–92. doi: 10.1111/j.1365-2435.2012.02040.x

Love, O. P., Rilett, A., and Capelle, P. M. (n.d.). Maternal stress modulates the effects of rearing environment enrichment on reintroduction phenotypes in Chinook salmon. *Conserv. Physiol.*

Lutterschmidt, W. I., and Hutchison, V. H. (1997). The critical thermal maximum: History and critique. *Can. J. Zool.* 75, 1561–1574. doi: 10.1139/z97-783

Mantua, N., Tohver, I., and Hamlet, A. (2010). Climate change impacts on streamflow extremes and summertime stream temperature and their possible consequences for freshwater salmon habitat in Washington State. *Clim. Change* 102, 187–223. doi: 10.1007/s10584-010-9845-2

Marshall, D. J., and Uller, T. (2007). When is a maternal effect adaptive? *Oikos* 116, 1957–1963. doi: 10.1111/j.2007.0030-1299.16203.x

Martins, E. G., Hinch, S. G., Patterson, D. A., Hague, M. J., Cooke, S. J., Miller, K. M., et al. (2012). High river temperature reduces survival of sockeye salmon (*Oncorhynchus nerka*) approaching spawning grounds and exacerbates female mortality. *Can. J. Fish. Aquat. Sci.* 69, 330–34212. doi: 10.1139/F2011-154

McCullough, D. A. (1999). *A Review and Synthesis of Effects of Alterations to the Water Temperature Regime on Freshwater Life Stages of Salmonids, with Special Reference to Chinook Salmon*. Seattle: U.S. Environmental Protection Agency.

McCullough, D. A., Bartholow, J. M., Jager, H. I., Beschta, R. L., Cheslak, E. F., Deas, M. L., et al. (2009). Research in thermal biology: burning questions for coldwater stream fishes. *Rev. Fish. Sci.* 17, 90–115. doi: 10.1080/10641260802590152

McDonnell, L. H., and Chapman, L. J. (2015). At the edge of the thermal window: effects of elevated temperature on the resting metabolism, hypoxia tolerance and upper critical thermal limit of a widespread African cichlid. *Conserv. Physiol.* 3, 1–13. doi: 10.1093/conphys/cov050

Messmer, V., Pratchett, M. S., Hoey, A. S., Tobin, A. J., Coker, D. J., Cooke, S. J., et al. (2017). Global warming may disproportionately affect larger adults in a predatory coral reef fish. *Glob. Chang. Biol.* 23, 2230–2240. doi: 10.1111/gcb.13552

Meylan, S., Miles, D. B., and Clobert, J. (2012). Hormonally mediated maternal effects, individual strategy and global change. *Philos. Trans. R. Soc. L. B Biol. Sci.* 367, 1647–1664. doi: 10.1098/rstb.2012.0020

Mousseau, T. A., and Fox, C. W. (1998). Maternal effects as adaptation. *Trends Ecol. Evol.* 13, 403–407. doi: 10.1017/CBO9781107415324.004

Munday, P. L. (2014). Transgenerational acclimation of fishes to climate change and ocean acidification. *F1000Prime Rep.* 6, 1–7. doi: 10.12703/p6-99

Muñoz, N. J., Farrell, A. P., Heath, J. W., and Neff, B. D. (2017). Hematocrit is associated with thermal tolerance and modulated by developmental temperature in juvenile chinook salmon. *Physiol. Biochem. Zool.* 91, 757–762. doi: 10.1086/695556

Nakagawa, S., and Schielzeth, H. (2013). A general and simple method for obtaining R2 from generalized linear mixed-effects models. *Methods Ecol. Evol.* 4, 133–142. doi: 10.1111/j.2041-210x.2012.00261.x

Neuheimer, A. B., and Taggart, C. T. (2007). The growing degree-day and fish size-at-age: the overlooked metric. *Can. J. Fish. Aquat. Sci.* 64, 375–385. doi: 10.1139/F07-003

Osborne, J. W. (2010). Improving your data transformations: applying the Box-Cox transformation. *Pract. Assess. Res. Eval.* 15, 1–9.

Pérez-Casanova, J. C., Rise, M. L., Dixon, B., Afonso, L. O. B., Hall, J. R., Johnson, S. C., et al. (2008). The immune and stress responses of Atlantic cod to long-term increases in water temperature. *Fish Shellfish Immun.* 24, 600–609. doi: 10.1016/j.fsi.2008.01.012

Piersma, T., and Drent, J. (2003). Phenotypic flexibility and the evolution of organismal design. *Trends Ecol. Evol.* 18, 228–233. doi: 10.1016/S0169-5347(03)00036-3

Pike, N. (2011). Using false discovery rates for multiple comparisons in ecology and evolution. *Methods Ecol. Evol.* 2, 278–282. doi: 10.1111/j.2041-210X.2010.00061.x

Polakof, S., Panserat, S., Soengas, J. L., and Moon, T. W. (2012). Glucose metabolism in fish: a review. *J. Comp. Physiol. B* 182, 1015–1045. doi: 10.1007/s00360-012-0658-7

R Core Team (2018). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: http://www.R-project.org/

Rahmstorf, S., and Coumou, D. (2011). Increase of extreme events in a warming world. *PNAS* 109, 17905–17909. doi: 10.1073/pnas.1114368109

Rohr, J. R., and Palmer, B. D. (2013). Climate change, multiple stressors, and the decline of ectotherms. *Conserv. Biol.* 27, 741–751. doi: 10.1111/cobi.12086

Roth, O., Beemelmanns, A., Barribeau, S. M., and Sadd, B. M. (2018). Recent advances in vertebrate and invertebrate transgenerational immunity in the light of ecology and evolution. *Heredity* 121, 225–238. doi: 10.1038/s41437-018-0101-2

Ruuskanen, S. (2015). Hormonally-mediated maternal effects in birds: lessons from the flycatcher model system. *Gen. Comp. Endocrinol.* 224, 283–293. doi: 10.1016/j.ygcen.2015.09.016

Ryu, T., Veilleux, H. D., Donelson, J. M., Munday, P. L., and Ravasi, T. (2018). The epigenetic landscape of transgenerational acclimation to ocean warming. *Nat. Clim. Change* 8, 504–509. doi: 10.1038/s41558-018-0159-0

Sandblom, E., Clark, T. D., Gräns, A., Ekström, A., Brijs, J., Sundström, L. F., et al. (2016). Physiological constraints to climate warming in fish follow principles of plastic floors and concrete ceilings. *Nat. Commun.* 7, 1–8. doi: 10.1038/ncomms11447

Schreck, C. B., and Tort, L. (2016). *The Concept of Stress in Fish*, 1 Edn. Amsterdam: Elsevier Inc.

Seebacher, F., White, C. R., and Franklin, C. E. (2014). Physiological plasticity increases resilience of ectothermic animals to climate change. *Nat. Clim. Chang.* 5, 61–66. doi: 10.1038/nclimate2457

Sheridan, J. A., and Bickford, D. (2011). Shrinking body size as an ecological response to climate change. *Nat. Clim. Chang.* 1, 401–406. doi: 10.1038/nclimate1259

Sheriff, M. J., Bell, A., Boonstra, R., Dantzer, B., Lavergne, S. G., McGhee, K. E., et al. (2017). Integrating ecological and evolutionary context in the study of maternal stress. *Integr. Comp. Biol.* 57, 437–449. doi: 10.1093/icb/icx105

Sheriff, M. J., and Love, O. P. (2013). Determining the adaptive potential of maternal stress. *Ecol. Lett.* 16, 271–280. doi: 10.1111/ele.12042

Sinervo, B. (1990). The evolution of maternal investment in lizards: an experimental and comparative analysis of egg size and its effects on offspring performance. *Evolution* 44, 279–294. doi: 10.2307/2409407

Sloman, K. A. (2010). Exposure of ova to cortisol pre-fertilisation affects subsequent behaviour and physiology of brown trout. *Horm. Behav.* 58, 433–439. doi: 10.1016/j.yhbeh.2010.05.010

Solomon, S., Qin, D., Manning, M., Marquis, M., Averyt, K., Tignor, M. M. B., et al. (eds) (2012). *Climate Change 2007: The Physical Science Basis Working Group I Contribution to the Fourth Assessment Report of the Intergovernment on Climate Change*. Geneva: IPCC.

Sopinka, N. M., Capelle, P. M., Semeniuk, C. A. D., and Love, O. P. (2017). Glucocorticoids in fish eggs: variation, interactions with the environment, and the potential to shape offspring fitness. *Physiol. Biochem. Zool.* 90, 15–33. doi: 10.1086/689994

Sopinka, N. M., Hinch, S. G., Healy, S. J., Raby, G. D., and Patterson, D. A. (2016). Effects of experimentally elevated egg cortisol on offspring traits in two species of wild Pacific salmon. *Environ. Biol. Fishes* 99, 717–728. doi: 10.1007/s10641-016-0513-x

Tunnah, L., Currie, S., and MacCormack, T. J. (2016). Do prior diel thermal cycles influence the physiological response of Atlantic salmon (Salmo salar) to subsequent heat stress? *Can. J. Fish. Aquat. Sci.* 139, 1–13. doi: 10.1139/cjfas-2016-0157

Uller, T., Nakagawa, S., and English, S. (2013). Weak evidence for anticipatory parental effects in plants and animals. *J. Evol. Biol.* 26, 2161–2170. doi: 10.1111/jeb.12212

van Vliet, M. T. H., Ludwig, F., and Kabat, P. (2013). Global streamflow and thermal habitats of freshwater fishes under climate change. *Clim. Change* 121, 739–754. doi: 10.1007/s10584-013-0976-0

Venables, W. N., and Ripley, B. D. (2003). Modern applied statistics with S. *Technometrics* 45, 1–495. doi: 10.1198/tech.2003.s33

Verhoeven, K. J. F., Simonsen, K. L., and McIntyre, L. (2005). Implementing false discovery rate control: increasing your power. *Oikos* 108, 643–647. doi: 10.1111/j.0030-1299.2005.13727.x

Warriner, T. R. (2019). *Prenatal Stress As a Potential Modulator of Offspring Survival, Phenotype, and Performance in Response to Elevated Temperatures*. master's thesis, University of Windsor, Windsor, ON.

Warriner, T. R., Semeniuk, C. A. D., Pitcher, T. E., and Love, O. P. (2020). Exposure to exogenous egg cortisol does not rescue juvenile Chinook salmon body size, condition, or survival from the effects of elevated water temperatures. *Ecol. Evol.* 10, 2466–2477. doi: 10.1002/ece3.6073

Wells, R. M. G., and Pankhurst, N. W. (1999). Evaluation of simple instruments for the measurement of blood glucose and lactate, and plasma protein as stress indicators in fish. *J. World Aquac. Soc.* 30, 276–284. doi: 10.1111/j.1749-7345.1999.tb00876.x

Wendelaar-Bonga, S. E. (1997). The stress response in fish. *Physiol. Rev.* 77, 591–625. doi: 10.1152/physrev.1997.77.3.591

Whitney, C. K., Hinch, S. G., and Patterson, D. A. (2014). Population origin and water temperature affect development timing in embryonic Sockeye salmon. *Trans. Am. Fish. Soc.* 143, 1316–1329. doi: 10.1080/00028487.2014.935481

Wingfield, J. C. (2013). Ecological processes and the ecology of stress: the impacts of abiotic environmental factors. *Funct. Ecol.* 27, 37–44. doi: 10.1111/1365-2435.12039

Woodward, G., Bonada, N., Brown, L. E., Death, R. G., Durance, I., Gray, C., et al. (2016). The effects of climatic fluctuations and extreme events on running water ecosystems. *Philos. Trans. R. Soc. B* 371:20150274. doi: 10.1098/rstb.2015.0274

Yin, J., Zhou, M., Lin, Z., Li, Q. Q., and Zhang, Y. Y. (2019). Transgenerational effects benefit offspring across diverse environments: a meta-analysis in plants and animals. *Ecol. Lett.* 22, 1976–1986. doi: 10.1111/ele.13373

Zhang, L., Zhao, Y., Hein-Griggs, D., and Ciborowski, J. J. (2018). Projected monthly temperature changes of the Great Lakes Basin. *Environ. Res.* 167, 453–467. doi: 10.1016/j.envres.2018.08.017

Zhang, Y., and Kieffer, J. D. (2014). Critical thermal maximum (CTmax) and hematology of shortnose sturgeons (*Acipenser brevirostrum*) acclimated to three temperatures. *Can. J. Zool.* 92, 215–221. doi: 10.1139/cjz-2013-0223

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REVIEW
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Transgenerational Plasticity and Bet-Hedging: A Framework for Reaction Norm Evolution

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Decision-making under uncertain conditions favors bet-hedging (avoidance of fitness variance), whereas predictable environments favor phenotypic plasticity. However, entirely predictable or entirely unpredictable conditions are rarely found in nature. Intermediate strategies are required when the time lag between information sensing and phenotype induction is large (e.g., transgenerational plasticity) and when cues are only partially predictive of future conditions. Nevertheless, current theory regards plasticity and bet-hedging as distinct entities. We here develop a unifying framework: based on traits with binary outcomes like seed germination or diapause incidence we clarify that diversified bet-hedging (risk-spreading among one's offspring) and transgenerational plasticity are mutually exclusive strategies, arising from opposing changes in reaction norms (allocating phenotypic variance among or within environments). We further explain the relationship of this continuum with arithmetic mean maximization vs. conservative bet-hedging (a risk-avoidance strategy), and canalization vs. phenotypic variance in a three-dimensional continuum of reaction norm evolution. We discuss under which scenarios costs and limits may constrain the evolution of reaction norm shapes.

Keywords: phenotypic plasticity, trans-generational plasticity, bet-hedging, coin-flipping, gene-by-environment interaction, canalization, adaptation, climate change

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INTRODUCTION

Changing conditions can promote evolutionary change in various ways (Botero et al., 2015; Tufto, 2015). One commonly envisioned mode of evolution is the continuous change of trait means as result of changing mean conditions (Darwin, 1859). Yet, although trait changes in response to novel conditions are widely observed (e.g., due to climate change, Piao et al., 2019), they frequently result from phenotypic plasticity (Boutin and Lane, 2014), i.e., changes of the phenotype in response to an environmental cue. Phenotypic plasticity may provide a short-term relief from changing conditions (Charmantier et al., 2008; Chevin et al., 2010), but also shield a genotype from selection and thereby prevent evolution (Oostra et al., 2018), or it may facilitate evolution via genetic accommodation (Kelly, 2019). In any case, phenotypic plasticity is a pervasive evolutionary strategy, and considered a major factor in a rapidly changing climate (Fox et al., 2019).

The time scale of phenotypic change depends on the time scale of environmental fluctuation (Rando and Verstrepen, 2007; Stomp et al., 2008). Fluctuations over very rapid timescales can be addressed by reversible plasticity, which includes, for example, the induction of plant defense when herbivores are present (Green and Ryan, 1972). Gradual long-term changes, on the other hand, are addressed by genetic adaptation. Between those extremes lie environmental fluctuations that are

roughly on the scale of one life span. When environments change over the course of an organism's development, they can be tackled by irreversible developmental plasticity, i.e., plastic adjustment of developmental pathways that lead to alternative phenotypes (Botero et al., 2015). For example, some *Daphnia* can produce protective phenotypes when chemical cues from predators are sensed during development (Krueger and Dodson, 1981). When environments are constant throughout an organism's life time but change from one generation to the next, phenotypic change can be induced in the offspring generation. These are referred to as anticipatory parental effects (Burgess and Marshall, 2014) or intergenerational inheritance (Perez and Lehner, 2019). For example, aphids that live under crowded conditions may produce winged offspring that can leave the colony and avoid high predation pressure or plant deterioration (Braendle et al., 2006). Lastly, when environmental fluctuations last for several generations, epigenetic modifications may be integrated into the germ line and affect multiple succeeding generations. This is referred to as transgenerational plasticity or non-genetic inheritance (Perez and Lehner, 2019; Adrian-Kalchhauser et al., 2020). For the remainder of the article we will refer to all these irreversible changes simply as phenotypic plasticity, ignoring the potential physiological constrains that may limit their evolution. They all have in common that there is a long delay between information sensing and phenotype induction.

Although often assumed, phenotypic plasticity does not need to be adaptive (Ghalambor et al., 2007; Arnold et al., 2019). Plasticity requires some environmental cue on which the induction of phenotypic change is based, and uncertainty around the future environmental state may turn plasticity maladaptive (Burgess and Marshall, 2014; Donelson et al., 2018). Such unpredictable conditions instead favor bet-hedging, which refers to the reduction of fitness variance (Cohen, 1966; Seger and Brockmann, 1987; Starrfelt and Kokko, 2012). Bet-hedging can be achieved by avoiding risky investments (conservative bet-hedging), or by spreading the risk among one's offspring (diversified bet-hedging), i.e., producing offspring with varying phenotypes (Seger and Brockmann, 1987; Starrfelt and Kokko, 2012). Although empirical evidence is difficult to obtain (Simons, 2011), bet-hedging is a likely explanation for high trait variance or unexpected trait means in many systems, such as the seed dormancy of desert annuals (Cohen, 1966), diapausing strategies of insects (Hopper, 1999) and annual killifish (Furness et al., 2015), wing dimorphisms (Grantham et al., 2016), facultative sexual reproduction (Gerber and Kokko, 2018), dispersal and partial migration (Goossens et al., 2020).

At fluctuations of intermediate time scales where there is a delay between information sensing and phenotype induction, both phenotypic plasticity (e.g., Baker et al., 2019) and bet-hedging (e.g., Venable, 2007) may be expected to evolve. Various theoretical studies have clarified the conditions that may lead to one or the other (Botero et al., 2015; Tufto, 2015), but although occurring potentially simultaneously, bet-hedging and plasticity are nevertheless often treated independently (Donelson et al., 2018). Moreover, when diversified bet-hedging and plasticity are considered jointly, there is no clear consensus about their exact relationship. Adaptive offspring variance that is needed for diversified bet-hedging might be either established by developmental instability (Simons and Johnston, 1997; Kærn et al., 2005; Veening et al., 2008; Woods, 2014; Dueck et al., 2016; Perrin, 2016) or by overly relying on cues with little predictive power ("microplasticity," Simons and Johnston, 2006; "hyperplasticity," Scheiner and Holt, 2012). With this article we aim to clarify the relationship between bet-hedging and plasticity, with special attention to readers that are familiar with plasticity but less familiar with bet-hedging theory. We will first use one simple numerical example (insect diapause) to explain the relationship of diversified bet-hedging, conservative bet-hedging and arithmetic mean maximization in detail. We will then extend the consideration to a range of environments whose state is partially predictable, thereby adding the potential for phenotypic plasticity. Lastly, we generalize from our example and describe a method to quantify phenotypic plasticity and bet-hedging based on reaction norm shapes.

AN EXAMPLE

Common examples of bet-hedging are transgenerational biphenisms, i.e., the parent decides among two possible physiological states of the offspring in the face of uncertainty (e.g., Cohen, 1966; Grantham et al., 2016; Maxwell and Magwene, 2017; see Simons, 2011 for further examples). One of these examples is the timing of insect diapause (Halkett et al., 2004; Pélisson et al., 2013), which we will use to illustrate the theory throughout this article.

Multivoltine insects benefit from exponential population growth throughout the growing season, but need to produce an overwintering (diapausing) generation before the onset of cold weather (Kivelä et al., 2016). Aphids, for example, reproduce by parthenogenesis during summer, which enables particularly quick population growth; in autumn they invest in sexual offspring that produce diapausing eggs, as frost kills the soft-bodied insects and only eggs survive (Simon et al., 2002). The struggle to keep the growing season long on one hand and to avoid death on the other hand puts diapause timing under intense selection pressure. If the onset of frost would be invariant, day length could be used as reliable cue of impeding winter, so plasticity in response to day length is expected to evolve. However, if just one generation faces early frosts, all offspring may simultaneously die and the genotype is driven to extinction, regardless of their otherwise high growth rates. Under unpredictable or only partially predictable conditions, bet-hedging strategies may therefore be expected to evolve (Halkett et al., 2004).

For the remainder of this article we will use examples that are loosely based on aphid overwintering. We will assume that parthenogenetic offspring (P_1) may produce four offspring when environmental conditions are mild, but face a 90% mortality rate when conditions change. In contrast, diapausing offspring (P_2) only replace themselves with 1 offspring in either environment. Hence we assign phenotype P_1 a fitness value of 4 in E_1 (summer), but only 0.1 in E_2 (winter), whereas phenotype P_2 achieves 1

fitness in either environment. We assume that the evolution of these growth rates is constrained, so only the proportion of each phenotype may evolve.

ARITHMETIC MEAN MAXIMIZATION, DIVERSIFIED BET-HEDGING AND CONSERVATIVE BET-HEDGING

We wish to explain the bet-hedging concept in detail with a few numerical examples. We first consider an entirely unpredictable environment, in which an aphid mother cannot collect any information about the potential environment of their offspring, i.e., there is a 50% chance that the offspring will face beneficial summer conditions (E_1), but also a 50% chance for harsh winter conditions (E_2). A genotype that invests exclusively in parthenogenesis (P_1) achieves on average 2.05 fitness (**Table 1**), while increasing the proportion of diapausing offspring (P_2) lowers arithmetic mean fitness. Nevertheless, a genotype that invests exclusively in diapause (P_2) is more successful on the long term, because the parthenogenetic genotype nearly dies out every two years. For example, a parthenogenetic population would decline to 16% of its original size over four years (4 * 0.1 * 4 * 0.1), while the population size of the diapausing genotype would remain constant. The arithmetic mean obviously fails here as predictor of long-term population growth.

If there are multiple decisions to make and the outcome is multiplicative, the geometric mean is a much better predictor for long-term growth, because it is sensitive to variance among years (Cohen, 1966; Seger and Brockmann, 1987; Starrfelt and Kokko, 2012). In the above example of population growth over multiple years, the lower arithmetic mean fitness was more than compensated by the reduction in fitness variance, therefore the risk-averse strategy achieved higher geometric mean fitness than the arithmetic mean maximization (AMM) strategy. This risk-aversive strategy of investing in lower fitness fluctuation at the cost of arithmetic mean fitness is called conservative bet-hedging (CBH), akin to investing in gold when stock markets fluctuate. The risky strategy of maximizing arithmetic mean fitness (AMM), on the other hand, is superior when fluctuations are low, and an analogy in economics would be the investment in a highly profitable product that is not insured against loss ("unhedged").

Now let us consider a genotype with high developmental instability, i.e., whose offspring phenotype is randomly determined (**Table 1**). This means that the arithmetic mean fitness is not reduced as strongly as that of the risk-aversive phenotype (100% P_2), but the fitness fluctuation between E_1 and E_2 is also not as great as that of the arithmetic mean maximizer (100% P_1). This genotype will increase in population size over four years by the factor 1.89 (2.5 * 0.55 * 2.5 * 0.55), so in this example it is clearly superior to both CBH and AMM. Investing equally in both phenotypes (P_1 and P_2) breaks down the fitness correlation among the offspring, as half of the offspring takes a risk, while the other half plays it safe (Starrfelt and Kokko, 2012). This strategy is similar to investing in a portfolio of stocks rather than a single stock and is called diversified bet-hedging (DBH).

The geometric mean can be calculated for any phenotype proportion p (proportion of P_2) between 0 and 100% (**Figure 1A**, solid blue line), showing that actually neither of the three strategies (AMM, CBH, DBH) is optimal. Instead, p = 0.61, i.e., a mix of CBH and DBH, yields the highest geometric mean fitness (**Table 1**). Starrfelt and Kokko (2012) explored the relationship among AMM, CBH and DBH in great detail, and explained fitness optimization as a three-way trade-off between maximizing the arithmetic mean, reducing fitness variance, and reducing fitness correlation among the offspring. However, as outlined in our example, this three-way relationship breaks down to a simple linear gradient when there are exactly two phenotypes to choose from.

The same principles also apply when the two environments do not occur with equal frequency, e.g., when the probability of E_2 (winter) is reduced to 20%. In this case the arithmetic mean fitness of P_1 and P_2 needs to be weighted by the frequencies of E_1 and E_2. Nevertheless, arithmetic mean fitness is still a linear function of the phenotype proportion p (**Figure 1A**, dashed orange line), and increasing the proportion of P_2 constitutes a change from AMM towards DBH or CBH. In this example with only occasionally adverse conditions, the optimum lies at p = 0.17 (solid orange line), i.e., much closer to an AMM strategy. If the frequency of E_2 is raised to 70%, on the other hand, the optimal strategy moves with p = 0.90 close to pure CBH (not shown). The optimal strategy thus strongly depends on the environmental frequency.

We wish to complete this description of fitness maximization in a single environment with two last special cases. First, we

TABLE 1 | Growth rate calculations for various phenotype proportions in a two-environment system.

	Proportion of P_2 (p)			
	0	**0.5**	**1**	**0.61**
E_1	**4** *(0 × 1 + 1 × 4)*	**2.5** *(0.5 × 1 + 0.5 × 4)*	**1** *(1 × 1 + 0 × 4)*	**2.17** *(0.61 × 1 + 0.39 × 4)*
E_2	**0.1** *(0 × 1 + 1 × 0.1)*	**0.55** *(0.5 × 1 + 0.5 × 0.1)*	**1** *(1 × 1 + 0 × 0.1)*	**0.65** *(0.61 × 1 + 0.39 × 0.1)*
Arithmetic mean	2.05	1.53	1	1.41
Geometric mean	0.63	1.17	1	1.19

A genotype may invest in two different phenotypes, P_1 and P_2, with a fixed proportion p. P_1 has four offspring if in environment E_1, but 0.1 if in E_2; P_2 achieves 1 fitness in either environment. We show arithmetic and geometric mean fitness across environments (Environments E_1 and E_2 are chosen with probability 0.5), as well as their calculation (italics).

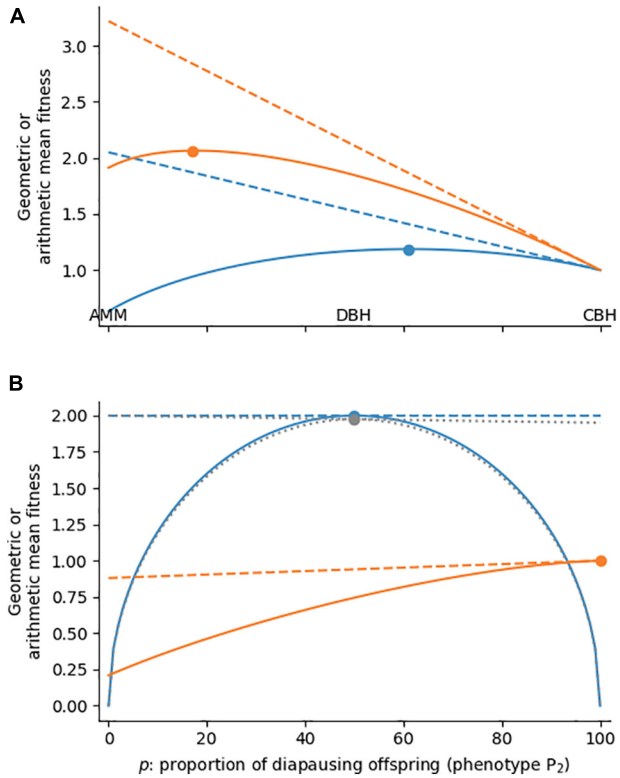

FIGURE 1 | Geometric (solid lines) and arithmetic mean fitness (dashed lines) when a genotype can express two discrete phenotypes in a two-state environment. **(A)** Conflict between geometric and arithmetic mean maximization. Environment E_2 (e.g., winter) occurs with frequencies of 0.5 (blue) or 0.2 (orange). Phenotype P_2 represents a risk-averse phenotype (e.g., diapausing offspring) with 1 fitness in either environment, the alternative phenotype is a phenotype with higher arithmetic mean fitness (4 fitness in E_1, 0.1 in E_2). **(B)** No or little conflict between arithmetic and geometric mean maximization. Blue line: E_2 occurs with frequency 0.5 and P_1 and P_2 are specialists for E_1 and E_2, respectively (4 fitness if matched, 0 fitness if mismatched); gray: same as blue line, but P_2 has 3.9 fitness in E_2; orange: fitness is the same as in panel **(A)**, but E_2 occurs with frequency 0.8. Colored dots represent the maxima of the respective functions.

consider the production of two specialist phenotypes, in which P_1 achieves a fitness of 4 in E_1, but none in E_2, while P_2 achieves 0 fitness in E_1 but 4 fitness in E_2 (thus deviating from the aphid example). With these parameters geometric mean fitness peaks at $p = 0.5$ (**Figure 1B**, blue solid line), so a strategy that maximizes developmental instability is optimal. Yet, the mixed production of offspring does not constitute DBH, because the diversification does not come at the cost of arithmetic mean fitness (i.e., the dashed blue line is flat). If, however, the growth rates of the two phenotypes are slightly uneven, e.g., reduced to 3.9 for P_2 in E_2, the same investment in P_2 would lower arithmetic mean fitness (dotted gray lines), and hence technically classify as a diversified bet-hedging strategy. This borderline example shows that the classification of bet-hedging strategies is not only a question of whether arithmetic mean fitness is reduced, but rather by how much. The second special case concerns very high probabilities of adverse conditions. When the frequency of E_2 is raised to 0.9, it carries so much weight that the arithmetic mean fitness does not decrease, but increase with the proportion of P_2 (**Figure 1B**, dashed orange line). The strategy that avoids variance is hence also the one which maximizes arithmetic mean fitness, so increasing geometric mean fitness (solid orange line) does not come at the cost of arithmetic mean fitness and CBH becomes impossible. In general, the linear gradient from AMM over DBH to CBH (and, in fact, the occurrence of bet-hedging) breaks down, when there is no conflict between arithmetic mean maximization and reduction of fitness variance. We will avoid these special situations in the remainder of the article.

CALCULATING OPTIMAL REACTION NORM SHAPES

We so far discussed the optimal phenotype proportion in a single, isolated environment. However, the benefit of diapause lies in adapting to a continually changing environment. Like in many other insects, aphid diapause is mainly governed by night length. Aphids exclusively reproduce by parthenogenesis under long-day (short night) conditions, but transition to the production of sexual forms under long-night conditions (Marcovitch, 1923). The diapause decision can hence be visualized as a biphenic reaction norm, in which the x-axis represents a continuous night length and the y-axis represents a probability (or, from the mother's perspective, a proportion) of diapause induction between 0 and 100%. This reaction norm to night length generally follows a logit-curve that ranges from a probability of zero under short nights to a probability of 1 under long nights, and the inflection point at which half of the offspring are diapausing forms is called critical day length (Danilevskii, 1965). The night length response is additionally modulated by temperature (warm temperatures delay diapause), but we ignore the additional plasticity in response to temperature in our considerations.

We will now use the diapause example to illustrate how to calculate optimal reaction norm shapes. Imagine an environment in which winter onsets over many years always occur at 14 h night length. Obviously night length would be a reliable cue and plasticity in response to night length can be expected to evolve. Conversely, night length is useless as cue for a plastic response if winter onset fluctuates randomly. Between those extremes lies an only partially reliable cue, i.e., there is between-years variation in the relationship of night length and winter onset. For example, winter onset may in some years coincide with a night length of 14 h, but fall in other years on an earlier (13.8 h) or later (14.5 h) date, which can be described by a normal distribution with a mean of 14 h and some standard deviation. We now use three different scenarios of how environmental conditions (winter onset) may vary: 1) Winter onset fluctuates according to a normal distribution $N_1(14, 1)$ with a mean cue value of 14 h and standard deviation 1; 2) Winter onset follows a normal distribution $N_2(14, 4)$ with a mean cue value of 14 h and standard deviation 4, thus simulating lower predictability by night length; 3) Winter onset fluctuates according to a normal distribution $N_3(14, 2)$ with standard deviation 2, but half of the winters are

mild enough that offspring of type P_1 (e.g., parthenogenetic offspring) can survive.

The cumulative distribution function of N describes the probability that winter will occur at a night length of c or lower (**Figure 2A**). If, for example, an aphid lives in an environment of exactly 14 hours night length, it can expect that the offspring will experience winter conditions with a 50% probability (the optimal phenotype proportion is then 0.61, see **Table 1**). At 13 h night length winter onset is less probable (18%) for environment N_1 (blue line) than for N_2 (41%, orange line), because winter onset variability is lower. In N_3 the probability distribution must be multiplied by 0.5, i.e., with the chance that winter is mild (green line). This reduces the probability of winter onset at c = 13 h to 16%. Given these environmental frequencies and the fitness values introduced earlier (parthenogenesis: 4/0.1; diapause: 1/1; in summer/winter conditions, respectively), one can now calculate the optimal proportion p as described in section "Arithmetic Mean Maximization, Diversified Bet-Hedging and Conservative Bet-Hedging." This proportion is 0.47 (nearly pure DBH) in scenario 1, as there is considerable risk of unfavorable conditions, but in scenarios 2 and 3 the ratios drop to 0.12 and 0.11, respectively. Thus, DBH is favored over pure AMM with increasing probability of winter conditions. The same calculations can be performed along the whole range of c, so the complete optimal reaction norm can be calculated if mean and standard deviation of the environment-cue relationship are known (**Figures 2B,C**).

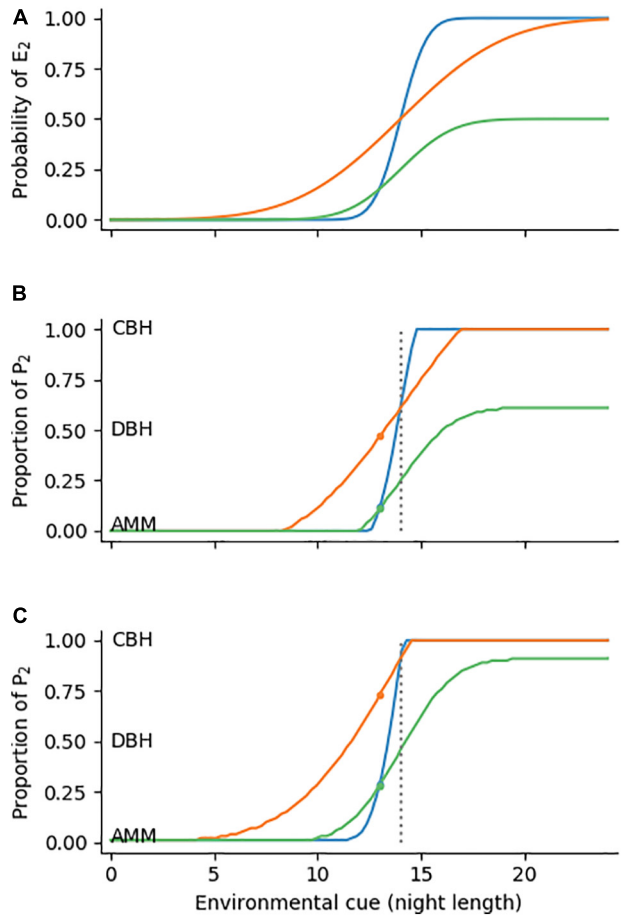

FIGURE 2 | Panel **(A)** Probability of encountering environment E_2 (winter conditions) for different values of an environmental cue c (e.g., night length). E_2 fluctuates around c according to three normal distributions $N_1(14,1)$, $N_2(14,4)$, and 0.5 * $N_3(14,2)$ (blue, orange, green). Shown are cumulative probability functions of the three distributions. **(B)** Optimal reaction norm shapes (e.g., proportion p of diapausing offspring for different night lengths) under the three scenarios of environmental uncertainty introduced in panel **(A)**. As in the main text, fitness of P_1 (parthenogenesis) is 4 in E_1 and 0.1 in E_2, whereas fitness of P_2 is always 1. **(C)** optimal reaction norm shapes when fitness of P_1 is 4/0 and fitness of P_2 is 1.8/1.8 in E_1/E_2, respectively. Dotted lines represent c = 14 h, small colored dots refer to the examples given in the main text.

With these considerations we explained the reaction norm shape as a series of binary decisions. In each of these decisions, phenotype proportions may range from AMM to CBH, with DBH in between. The overall degree of bet-hedging is hence defined by the reaction norm shape, and in our specific examples mostly correlates with the reaction norm slope (**Figure 2B**, orange and blue lines) and range (green line). However, as indicated by the skew in the orange line towards the lower range of c (AMM is discouraged even under low risk) in **Figure 2C**, more complex shapes are also possible and the relative contribution of each strategy is difficult to quantify. Furthermore, our examples are based on cumulative densities of normal distributions, but depending on the environmental cue, other shapes (e.g., bimodal, sinusoid) are possible. We hence require summary statistics that adequately describe the reaction norm shape.

CLASSIFICATION OF REACTION NORM SHAPES

In this section we will describe some typical reaction norm shapes and discuss useful summary statistics to describe the overall degree of plasticity, arithmetic mean maximization, conservative bet-hedging and diversified bet-hedging. First, let us assume a "plastic" reaction norm (**Figure 3A**, dark blue line). A step function describes a sudden switch from one phenotype (AMM) to the other (CBH), and the number of environments in which a mix of phenotypes is produced is minimized. This function maximizes the standard deviation of phenotype proportions p across environments. We refer to the variance of p as σ^2_{among}. The opposite of a step function is one in which the mother's decision is entirely independent of the environmental cue, i.e., left to developmental instability, and both phenotypes are produced in equal measure (DBH; **Figure 3A**, light blue line). While σ^2_{among} is zero, there is variance in phenotypes within each environment (σ^2_{within}). The trait choice is a Bernoulli draw and the variance of each p is calculated as p * (1 – p), so we define σ^2_{within} across environments as the mean Bernoulli variance. The two variance components (among and within environments) complement each other, and we define their sum $s = \sigma^2_{among} + \sigma^2_{within}$ as the phenotypic variance of the genotype. It is not possible to maximize both σ^2_{among} (steep slope, high range) and σ^2_{within}

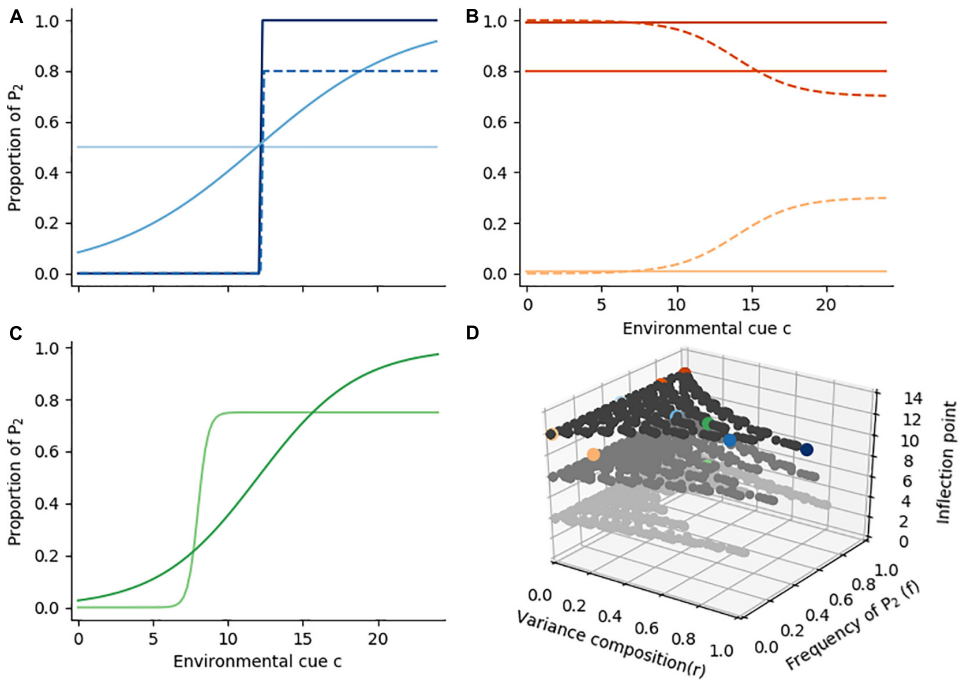

FIGURE 3 | Example reaction norm shapes. **(A)** Four reaction norm shapes that exhibit high phenotypic variance. Variance may occur exclusively among environments (dark blue), exclusively within each environment (light blue), or as a mix of both variance components (medium blue, solid and dashed). We refer to the ratio of the variance components (among : within) as *r*. **(B)** Three different reaction norms with $r = 0$ (solid lines), and two different reaction norms with $r = 0.14$ (dashed). The reaction norms differ in the mean frequency *f* of phenotype P_2, which also affects the phenotypic variance *s* (i.e., the sum of variance among and within environments). Reaction norms with $f = 0$ (light orange) and $f = 1$ (dark orange) are canalized ($s = 0$), and phenotypic variance is maximized at $f = 0.5$ (see panel **A**). **(C)** Two logistic reaction norms with the same *f* and *r*, but different inflection points. **(D)** possible parameter space of *r*, *f*, and inflection points. Gray dots depict sample reaction norms across the range of possible parameters (darkness scales with z-axis), colored dots indicated samples from panels **(A–C)** in their respective color.

(minimal departure from 50%) at once, but intermediate reaction norms with mixed contributions of σ^2_{among} and σ^2_{within} are possible (solid and dashed medium blue lines). The trade-off between σ^2_{among} and σ^2_{within} can be described by the ratio $r = \frac{\sigma^2_{among}}{\sigma^2_{within}}$. *r* thus describes the degree of developmental (in)stability across environments.

The variance composition is not the only parameter in which reaction norms may vary. Reaction norms may, for example, be flat ($r = 0$), but the proportion of P_2 (p) might be zero (**Figure 3B**, light orange line), 0.8 (dark orange) or 1 (darkest line) in all environments. These reaction norms differ in the mean frequency of phenotype P_2 across environments, which we denote as *f*. A frequency of zero indicates a pure AMM strategy, while $f = 1$ is a pure CBH strategy. A mean frequency of 0.5 indicates a reaction norm with maximal phenotypic variance (s), enabling the aforementioned gradient from phenotypic plasticity to DBH (**Figure 3A**, solid lines). As with **Figure 3A**, intermediate reaction norm shapes are also possible: a reaction norm may, for example, range from $p = 0$ to $p = 0.3$ or from $p = 0.7$ to $p = 1$ (**Figure 3B**, dashed lines). Reaction norms can thus vary from complete canalization to high phenotypic variance, and we express their shape by mean frequency of phenotype P_2 and by the variance composition. A canalized reaction norm may be only expressing risk-aversive phenotypes, or only expressing arithmetic mean optimizers, whereas high phenotypic variance may indicate steep plastic reaction norms or DBH.

The two shape parameters *f* and *r* reflect the reaction norm shape to a reasonable extent, but as summarizing statistics they cannot sufficiently describe all its features. For example, the reaction norms in **Figure 3C** both share the same mean frequency (0.5) and variance composition (0.47), but the strategies under environments that correspond to a low cue *c* differ considerably. In our aphid example these two strategies differ in the mean timing of diapause induction, which is an important consideration when the onset of seasons is under directional change (IPCC, 2014). This mean timing can be assessed by calculating the inflection point (called critical day length for diapause reaction norms), but for non-logistic reaction norms or more complicated reaction norm shapes a different approach, e.g., based on autocorrelation patterns, is required.

In summary we discussed three important parameters that describe a reaction norm shape: The frequency *f*, the variance composition *r* (among:within environments), and (for logistic reaction norms) the inflection points. These three parameters are partially interdependent of one another, and can be drawn as three perpendicular axes (**Figure 3D**; see also **Supplementary Figure S1** for an alternative representation). The resulting

parameter space has three distinct ends which conform to maximum plasticity (i.e., a step-function, dark blue dot), CBH (dark orange), and AMM (light orange). Parameters outside these bounds are not possible, e.g., DBH and plasticity cannot occur in canalized reaction norms, and on the other hand mean frequencies of 0.5 necessarily imply phenotypic variance by DBH or plasticity.

REACTION NORM EVOLUTION

So far we described optimal strategies in a single environment (Section "Arithmetic Mean Maximization, Diversified Bet-Hedging and Conservative Bet-Hedging"), calculated optimal reaction norm shapes (Section "Calculating Optimal Reaction Norm Shapes"), and explored which reaction norm shapes are generally possible (Section "Classification of Reaction Norm Shapes"). We now return to our aphid diapause example to illustrate how optimal reaction norms change when environmental conditions and fitness functions are altered. We will cover cases with more frost-resistant parthenogenetic forms (i.e., higher fitness of P_1 in E_2), harsher summer environments (lower fitness of P_1 in E_1), and three forms of change in the environment that are directly relevant for aphid biology: first, mean winter onset may vary with latitude, with earlier winter onset at high latitudes (Danilevskii, 1965). Secondly, winter onset dates may vary among years, which is the condition that should lead to bet-hedging in diapause timing (Halkett et al., 2004). Lastly, aphid populations in warmer climates frequently lost the ability to produce sexual forms and reproduce by parthenogenesis throughout the year (anholocyclic life cycles, Simon et al., 2002). The preparation for winter makes only sense if there is sufficient change in environmental conditions, so this kind of canalization (obligate development) is expected at southern latitudes.

We start with environments that vary in among-years predictability. Using night length (in hours) as a cue c, we consider scenarios where winter onset is normally distributed with a mean cue c of 14 and standard deviations ranging from 0 to 10. In our standard example with growth rates of 4/0.1 (parthenogenetic) and 1/1 (diapausing), the optimal mean frequency f of risk-aversive (diapausing) phenotypes increases with environmental variance (**Figure 4A**, blue solid line), while the variance ratio r (among : within environments) decreases (**Figure 4C**, blue solid line). Thus, a greater tendency towards DBH and CBH is expected to evolve across environments in unpredictable conditions (see also **Figure 4B**, blue lines). With decreasing growth rate of P_1 in E_1(parthenogenesis in summer) the optimal ratio decreases less sharply and the frequency of P_2 (diapause) increases more strongly (solid orange and green lines in **Figure 4A**, green lines in **Figure 4B**). Here the riskier strategy pays off less, and the balance is shifted towards CBH. When the growth rate of P_1 in E_2 (winter) is raised to 0.33 (frost tolerance) both r and f change less steeply with environmental unpredictability (dashed lines), i.e., the optimal reaction norms tend towards AMM. Increasing the growth rate in E_2 further to 0.66 (dash-dotted lines) leads to a strategy that ignores environmental risk, except when the chance of mild

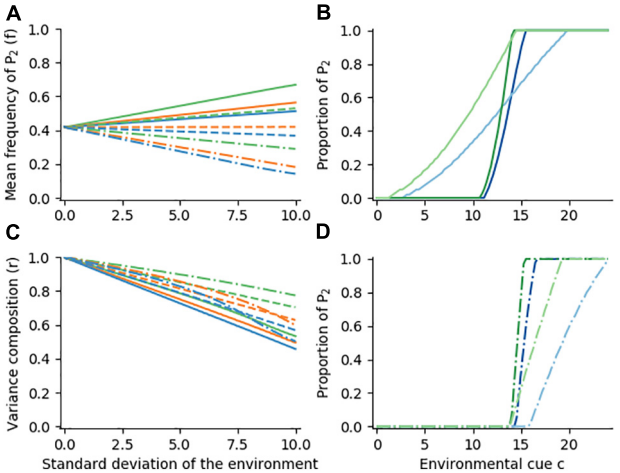

FIGURE 4 | Optimal reaction norm shapes for various growth rate functions and different levels of environmental predictability. Environments are normally distributed around a cue c with a mean of 14. Mean frequency f of phenotype P_2 (Panel **A**) and variance composition r (Panel **C**) are plotted against standard deviation of the environment. Growth rates of P_2 (diapause) are always 1 for both environments (summer and winter); growth rates of P_1 (parthenogenesis) in E_1/E_2 are 4/0.1 (blue, solid), 3/0.1 (orange, solid), 2/0.1 (green, solid); 4/0.33, 3/0.33, 2/0.33 (dashed blue, orange and green lines); and 4/0.66, 3/0.66, 2/0.66 (dash-dotted blue, orange and green lines). Panels **(B–D)** show optimal reaction norms for environments with standard deviations of 2 (darker shade) and 8 (lighter shade) in the according line styles and colors.

(summer) conditions is very low. The range of environments that feature a sufficiently low chance of P_1 decreases with increasing environmental variance, causing a drop of both f and r as a sign of canalization to AMM (**Figure 4D**). Overall, both CBH and DBH can be expected under unpredictable conditions, but their relative benefits vary depending on the arithmetic mean fitness of risk-aversive and risk-prone phenotypes.

We now simulate global changes in the probability of events, for instance increased or decreased probabilities of severe winters. For the latter, we multiply the normal distribution by 0.5, overall halving the probability of being in the harsh environment E_2 (see also **Figure 2A**). This discourages risk-aversion and, for example, having all offspring diapausing is no longer beneficial (**Figure 5**). When the growth rate of P_1 is either 4 (summer) or 0.1 (winter), the frequency f stagnates at 0.2 to 0.25, while the ratio r decreases from 0.47 to 0.17 (**Figures 5A,C**, solid blue line). This is because the reaction norm range is constrained (**Figure 5B**). A lower growth rate of P_1 in E_1 restores phenotypic variance (**Figures 5A,C**, orange and green lines), as it reduces its arithmetic mean fitness and makes the alternative phenotype again more profitable (**Figure 5B**, green lines). Lowering the environmental risk further increases the benefit of arithmetic mean maximization (dashed lines) and eventually leads to AMM under all environmental conditions (dash-dotted lines). Overall, **Figure 5** shows that a global reduction of the probability for E_2 may discourage CBH, and instead favor AMM. For example, a lower risk of freezing in winter may explain the existence of anholocyclic lines.

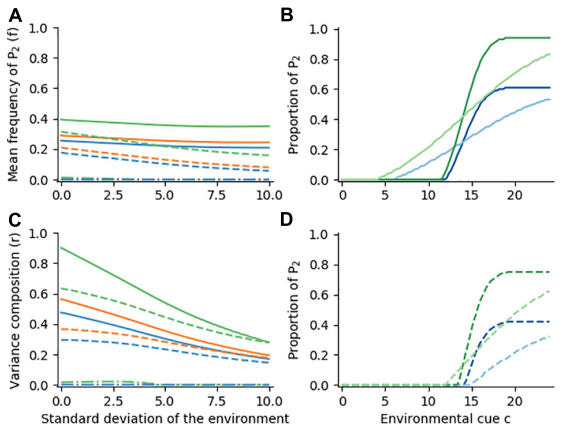

FIGURE 5 | Optimal reaction norm shapes for various growth rate functions and different levels of environmental predictability. Environments are normally distributed around a cue *c* with a mean of 14, but multiplied by 0.5. Panels, growth rates, coloring and line styles are the same as **Figure 4**.

A third axis of environmental variation concerns changes in mean environments. Moving the distribution of environments to a mean *c* of 9 h simulates the change of winter onset with latitude, as well as the effects of a changing climate. Although highly relevant for the optimization of fitness, the changes in optimal reaction norm shapes are trivial to describe. We refer to **Supplementary Material S2** for further exploration.

In general, we find that *r* and *f* evolve with changes in environmental predictability (**Figure 4**, solid lines), leading to CBH and DBH in unpredictable environments. Changes in the fitness function (growth rates in our example) may, however, affect the balance of AMM and CBH, and very low rewards for CBH instead lead to the evolution of risky strategies that seek to maximize the arithmetic mean (**Figure 4**, dash-dotted lines). When the probability of adverse conditions is globally lowered across the range of environments (e.g., mild winters), the reaction norm range can become constricted, which further affects the balance of the fitness maximization strategies. Lastly, *f* additionally depends strongly on the mean environment (e.g., winter onset, Supp. S2), but within reasonable limits the general shape of the reaction norms is not affected.

DISCUSSION

Phenotypic plasticity can help organisms adapt to changing conditions (Fox et al., 2019), but this requires a predictable cue (Bonamour et al., 2019). Especially for transgenerational plasticity cues are not entirely predictable (Burgess and Marshall, 2014; Donelson et al., 2018), which, at least under some conditions, favors bet-hedging instead (Botero et al., 2015; Tufto, 2015). Nevertheless, the value of bet-hedging strategies as alternatives to plasticity is frequently overlooked.

Starrfelt and Kokko (2012) have explained bet-hedging, including its mathematical foundation, in great detail. The main finding was that arithmetic mean fitness maximization, diversified bet-hedging and conservative bet-hedging form a three-way trade-off of conflicting strategies. However, it was difficult to see how these strategies play out in practice (Haaland et al., 2020). We provided a simple, detailed calculation of fitness based on insect diapause as example. Based on this system with only two possible phenotypes (biphenisms) we described how a conflict between arithmetic and geometric mean optimization can result in bet-hedging (**Figures 1A,B**). We explained that the three strategies form a gradient, in which arithmetic mean maximization (AMM) and conservative bet-hedging (CBH) are represented by distinct phenotypes, and diversified bet-hedging (DBH) by a mixture of the two extremes. We also extended the concept by adding a cue the organisms can respond to, thereby incorporating reaction norms and the potential for phenotypic plasticity. We identified the mean phenotype frequency *f* and the variance composition *r* as two summary statistics of reaction norms that allow distinguishing between AMM, CBH, DBH and plasticity, and the sum *s* of the variance components as a measure of phenotypic variance. Moreover, for logistic reaction norm shapes we discuss the inflection point as a third useful summary statistic.

Arithmetic Mean Maximization vs. Conservative Bet-Hedging

In section "Arithmetic Mean Maximization, Diversified Bet-hedging and Conservative Bet-hedging" we described AMM, DBH and CBH as a linear gradient of strategies to cope with a single environment. When extended to multiple environments, a flat reaction norm at $p = 0$ (**Figure 3B**, light orange line) maximizes arithmetic mean fitness (see also **Figure 1A**), and any adaptive deviation from this line incorporates some bet-hedging (in the cases we consider; see **Figure 1B** for exceptions). Thus, the mean phenotype frequency *f* is a direct measure of the degree of CBH in a reaction norm shape. We illustrated that *f* correlates with the frequency of the harsh environment E_2 (compare **Figure 4A** and **Supplementary Figure S1**, panel A), but *f* also changes with the degree of environmental variance: higher environmental risk shifts optimal reaction norms towards DBH and CBH (**Figure 4A**, solid lines; **Figure 4B**, dark blue vs. light blue lines), in line with expectations from other studies (Simons, 2011; Tufto, 2015). This shift is particularly noticeable when the potential fitness gain from a risk-prone strategy is low (**Figure 4B** green lines; **Figure 2C**, orange lines). If, on the other hand, the risk is reduced and the potential pay-off high (**Figure 4A, dashed** and **dot**-dashed lines; **Figure 5**), the optimal reaction norm shapes are shifted towards risk-prone (AMM) strategies (Halkett et al., 2004). Thus our framework made clear that arithmetic mean maximization and variance avoidance form exact opposites on a gradient of strategies that is reflected by *f* (**Figure 3D**, y-axis).

We have illustrated that frequencies or means of reaction norms that mismatch with environmental means might serve a function. Recent climate change imposes novel environmental conditions, and species or populations whose trait means do not evolve in concert with environmental means are often considered as under risk (e.g., Charmantier and Gienapp, 2014), ignoring that this phenotype-environment mismatch may in fact be due

to an adaptive CBH strategy. This is not to say that CBH can be invoked whenever environmental variance is observed (Simons, 2011), but any combination of mean maximization and variance avoidance (f) has the potential to be adaptive depending on life history and environmental variance.

Phenotypic Plasticity vs Diversified Bet-Hedging

Reaction norms that are not entirely canalized exhibit some degree of phenotypic plasticity and/or diversified bet-hedging (**Figures 3A,B,C**), and we expressed their relative contribution with the variance ratio r. When environmental cues convey reliable information, a high r is adaptive, i.e., phenotypes change with the environmental cues, but vary only little for any given cue (solid dark blue lines in **Figures 2B**, 3A, 4B; Botero et al., 2015; Tufto, 2015). This reaction norm pattern is commonly referred to as phenotypic plasticity, or, when the offspring phenotype is dictated by the (grand-) parental environment, as inter- or transgenerational plasticity (Perez and Lehner, 2019). A low r, on the other hand, corresponds to DBH across the range of possible environments (orange line in **Figure 2B**, solid light blue lines in **Figures 3A,B**), and occurs predominantly when cues convey little information about the optimal phenotype (Cohen, 1966). Our simple models based on aphid diapause illustrate such a negative relationship between r and cue variance for all but the most extreme growth rate functions (**Figures 4C**, 5C). We therefore see phenotypic plasticity and diversified bet-hedging as a continuum of evolutionary strategies that is based on the reaction norm shape (**Figure 3D**, x-axis).

This definition extends classical concepts of bet-hedging and transgenerational plasticity. Plasticity has a long history of being related to reaction norm shapes (Woltereck, 1913; Bradshaw, 1965), but diversified bet-hedging is not as easily visualized, nor is the relationship with plasticity entirely clear. On the one hand, developmental instability has been seen as a cause of diversified bet-hedging (Simons and Johnston, 1997; Kærn et al., 2005; Woods, 2014; Dueck et al., 2016; Perrin, 2016). Low copy numbers e.g., of transcriptional regulators (Volfson et al., 2006) cause sampling errors that ultimately lead to expression of alternative phenotypes. On the other hand, DBH might be produced by a reaction norm to noise ("microplasticity," Simons and Johnston, 2006; "hyperplasticity," Scheiner and Holt, 2012). For example, Maxwell and Magwene (2017) engineered a yeast model that evolved a response to estradiol, a compound that was entirely unrelated to fitness but ensured phenotypic variance in a fluctuating environment. Accordingly, the relationship between diversified bet-hedging and plasticity might be perceived as nested or as one of two competing strategies. We instead distinguish them as the two extremes on a continuum of strategies, that correspond to a continuum of reaction norm shapes.

Fixed vs. Flexible Development

The phenotype frequency f and the variance composition r are not entirely independent (**Figure 3D**), because phenotypic variance s, i.e., the sum of variance among and within environments, is a quadratic function of f: when f is zero (pure AMM or CBH, **Figure 3B**) there is no phenotypic variance and hence no potential for DBH or phenotypic plasticity. When f is 0.5, on the other hand, DBH, phenotypic plasticity, or a mix of the two strategies is necessarily required (**Figures 3A,D**).

In section "Reaction Norm Evolution" we altered the amplitude between summer and winter conditions, both by changing the fitness of the phenotypes (**Figure 4, green** and **orange** lines) and by affecting the global probability of E_2 (**Figure 5**). Reductions in the difference between summer and winter led to a reduction of phenotypic variance, i.e., to a decrease in f towards canalization (**Figures 4A**, **Figures 5B,D**), illustrating that phenotypic variance is not beneficial when environments are stable. The relationship between the variance composition r and environmental variance was, however, maintained (**Figure 5C**, dark vs. light lines in **Figure 5B**). The benefits of plasticity and DBH under predictable and unpredictable conditions, respectively, were thus also apparent under partially canalizing conditions.

Our examples clarified that phenotypic variance is a function of f in binomial reaction norms, and as such it is equally related to both phenotypic plasticity and diversified bet-hedging. The opposite of phenotypic variance (i.e., of plasticity and DBH) in our models is environmental canalization, a term which so far has been used ambiguously (Debat and David, 2001), as it was considered either the opposite of plasticity (Waddington, 1942; Van Buskirk and Steiner, 2009) or of developmental noise (Gibson and Wagner, 2000; Zhang and Hill, 2005) alone. Phenotypic plasticity is regarded an essential component of climate change adaptation (Fox et al., 2019), precisely because of the variance it entails; moreover, de-canalization by phenotypic plasticity may accelerate evolution through genetic accommodation (Kelly, 2019). We argue that the same mechanisms may apply for all modes of phenotypic variance, including diversified bet-hedging.

The Importance of Mean Timing

We introduced the inflection point as additional important reaction norm shape parameter (**Figure 3C**, z-axis in **Figure 3D**; **Supplementary Figure S1**). In our example the inflection point determined the mean timing of phenotypic change (i.e., the phenology), and clearly depended on the mean timing of environmental change (**Supplementary Figure S2**). The inflection point (called critical day length in diapause reaction norms) is known to change with latitude (Danilevskii, 1965; Bradshaw, 1976), and questions regarding its evolution are highly important under climate change (Saikkonen et al., 2012; Zohner et al., 2016). While limited to logistic reaction norms, we think the inflection point as reaction norm shape parameter deserves special attention, because many phenological traits are of binary nature (e.g., bird arrival, migration onset, plant germination and flowering) and hence modeled as logistic reaction norms.

Outlook

The world is simultaneously changing in climate means, variability and predictability (IPCC, 2014; Lenton et al., 2017; Bathiany et al., 2018), and there are many phenomenological

studies on responses to climate change (Parmesan and Yohe, 2003; Badeck et al., 2004; Cohen et al., 2018). However, only few detailed case-studies on the mechanisms of adaptation (Nussey et al., 2005; Gienapp et al., 2013; Lane et al., 2018) exist, and one cannot assume that a matching mean timing or a high level of plasticity is always adaptive (Boutin and Lane, 2014), just like one cannot assume CBH or DBH to be an optimal solution (Simons, 2011) – but one can analyze reaction norm shapes with the proposed shape parameters to decide whether it has the *potential* for adaptive tracking, arithmetic mean maximization, plasticity, bet-hedging or canalization (Joschinski and Bonte, 2020).

There is ample room to extend our framework. First of all, we focussed only on the *optimal* reaction norm shape. This ignores that CBH and DBH are often nearly equally suited strategies to cope with environmental uncertainty (Starrfelt and Kokko, 2012), i.e., the shape and curvature of the geometric mean fitness curve (**Figure 1A**) requires further consideration. Secondly, we have restricted our arguments to binary trans-generationally inherited traits, as these are commonly treated both empirically (Venable, 2007; Maxwell and Magwene, 2017; Scholl et al., 2020) and theoretically (Cohen, 1966; Halkett et al., 2004; Starrfelt and Kokko, 2012; Kivelä et al., 2016; Gerber and Kokko, 2018). For continuous traits, e.g., offspring size (Marshall et al., 2008), our calculations may not apply, because AMM, DBH and CBH need not lie on a linear gradient (i.e., intermediate trait values need not incur highest trait variance). Nevertheless, theory regarding Gaussian functions arrives at a similar conclusion: that offspring variance evolves to the amount of environmental mismatch that is not already covered by phenotypic plasticity (Tufto, 2015). This is equivalent to our finding that only the variance composition (r) changes with environmental variability, whereas the degree of phenotypic variance remains relatively constant (e.g., **Figure 5B**). Other possible extensions would include plastic responses that take place within an individual's life time. The opportunity for both within- and transgenerational plasticity may not only make one strategy obsolete (Luquet and Tariel, 2016), but also lead to complex interactions among the two (Fuxjäger et al., 2019). Similarly, fitness may include multiplicative instances within an individual's lifetime (e.g., iteroparity), shifting the balance from DBH towards CBH strategies, or conversely sum across generations ("fine-grained" environments), moving the balance towards AMM strategies (Haaland et al., 2020). Lastly, there are also potential bet-hedging strategies that appear entirely unrelated to transgenerational plasticity. These include, for example, an iteroparous life history (Garcia-Gonzalez et al., 2015), hotspots for genetic mutations ("contingency loci", Rando and Verstrepen, 2007), and sexual reproduction in general (Li et al., 2017). A unification with these alternative strategies might lead to a better understanding of adaptation to rapid climate change.

CONCLUSION

In this review we rephrased reaction norm evolution as a complex trade-off among four strategies. It is increasingly recognized that changes in climate extremes and in predictability are as important as changes in means (IPCC, 2014; Donelson et al., 2018) – focusing only on strategies to match the mean is hence not fruitful. For example, failure to shift mean phenology with climate change (Gienapp et al., 2013) is not problematic per se – it could be mitigated by concurrent changes in phenotypic variance. Similarly, the lack of both phenotypic plasticity and mean change may not have severe fitness consequences, if the lack of plasticity is mitigated by diversified bet-hedging. It is the combination along all three axes that defines fitness in a given environment.

DATA AVAILABILITY STATEMENT

All code necessary to produce the figures is included in the **Supplementary Material S3**.

AUTHOR CONTRIBUTIONS

JJ and DB developed the theory and contributed to the final version of the manuscript. JJ wrote the first draft. DB supervised the work. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.517183/full#supplementary-material

Supplementary Figure 1 | Possible parameter space of r and f for different inflection points. Grey dots depict sample reaction norms across the range of possible parameters (darkness scales with inflection point).

Supplementary Figure 2 | Optimal reaction norm shapes for various growth rate functions and different levels of environmental predictability. Environments are normally distributed around a cue c with a mean of 9 h, all other parameters, coloring and line styles are the same as **Figure 4**.

Supplementary Table 1 | Python code that was used to generate the figures in the manuscript.

REFERENCES

Adrian-Kalchhauser, I., Sultan, S. E., Shama, L. N. S., Spence-Jones, H., Tiso, S., Valsecchi, C. I. K., et al. (2020). Understanding "non-genetic" inheritance: insights from molecular-evolutionary crosstalk. *Trends Ecol. Evol.* (in press). doi: 10.1016/j.tree.2020.08.011

Arnold, P. A., Nicotra, A. B., and Kruuk, L. E. B. (2019). Sparse evidence for selection on phenotypic plasticity in response to temperature.

Philos. Trans. R. Soc. B 374:20180185. doi: 10.1098/rstb.2018.0185

Badeck, F. W., Bondeau, A., Bottcher, K., Doktor, D., Lucht, W., Schaber, J., et al. (2004). Responses of spring phenology to climate change. *New Phytol.* 162, 295–309. doi: 10.1111/j.1469-8137.2004.01059.x

Baker, B. H., Sultan, S. E., Lopez-Ichikawa, M., and Waterman, R. (2019). Transgenerational effects of parental light environment on progeny competitive performance and lifetime fitness. *Philos. Trans. R. Soc. B* 374:20180182. doi: 10.1098/rstb.2018.0182

Bathiany, S., Dakos, V., Scheffer, M., and Lenton, T. M. (2018). Climate models predict increasing temperature variability in poor countries. *Sci. Adv.* 4:eaar5809. doi: 10.1126/sciadv.aar5809

Bonamour, S., Chevin, L.-M., Charmantier, A., and Teplitsky, C. (2019). Phenotypic plasticity in response to climate change: the importance of cue variation. *Philos. Trans. R. Soc. B* 374:20180178. doi: 10.1098/rstb.2018.0178

Botero, C. A., Weissing, F. J., Wright, J., and Rubenstein, D. R. (2015). Evolutionary tipping points in the capacity to adapt to environmental change. *PNAS* 112, 184–189. doi: 10.1073/pnas.1408589111

Boutin, S., and Lane, J. E. (2014). Climate change and mammals: evolutionary versus plastic responses. *Evol. Appl.* 7, 29–41. doi: 10.1111/eva.12121

Bradshaw, A. D. (1965). Evolutionary significance of phenotypic plasticity in plants. *Adv. Gen.* 13, 115–155. doi: 10.1016/S0065-2660(08)60048-6

Bradshaw, W. E. (1976). Geography of photoperiodic response in diapausing mosquito. *Nature* 262, 384–386. doi: 10.1038/262384b0

Braendle, C., Davis, G. K., Brisson, J. A., and Stern, D. L. (2006). Wing dimorphism in aphids. *Heredity* 97, 192–199. doi: 10.1038/sj.hdy.6800863

Burgess, S. C., and Marshall, D. J. (2014). Adaptive parental effects: the importance of estimating environmental predictability and offspring fitness appropriately. *Oikos* 123, 769–776. doi: 10.1111/oik.01235

Charmantier, A., and Gienapp, P. (2014). Climate change and timing of avian breeding and migration: evolutionary versus plastic changes. *Evol. Appl.* 7, 15–28. doi: 10.1111/eva.12126

Charmantier, A., McCleery, R. H., Cole, L. R., Perrins, C., Kruuk, L. E. B., and Sheldon, B. C. (2008). Adaptive phenotypic plasticity in response to climate change in a wild bird population. *Science* 320, 800–803. doi: 10.1126/science.1157174

Chevin, L.-M., Lande, R., and Mace, G. M. (2010). Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. *PLoS Biol.* 8:e1000357. doi: 10.1371/journal.pbio.1000357

Cohen, D. (1966). Optimizing reproduction in a randomly varying environment. *J. Theor. Biol.* 12, 119–129. doi: 10.1016/0022-5193(66)90188-3

Cohen, J. M., Lajeunesse, M. J., and Rohr, J. R. (2018). A global synthesis of animal phenological responses to climate change. *Nat. Clim. Change* 8, 224–228. doi: 10.1038/s41558-018-0067-3

Danilevskii, A. S. (1965). *Photoperiodism and Seasonal Development of Insects*, 1st Edn. Edinburgh: Oliver & Boyd.

Darwin, C. (1859). *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life*. London: J. Murray.

Debat, V., and David, P. (2001). Mapping phenotypes: canalization, plasticity and developmental stability. *Trends Ecol. Evol.* 16, 555–561. doi: 10.1016/S0169-5347(01)02266-2

Donelson, J. M., Salinas, S., Munday, P. L., and Shama, L. N. S. (2018). Transgenerational plasticity and climate change experiments: where do we go from here? *Glob. Change Biol.* 24, 13–34. doi: 10.1111/gcb.13903

Dueck, H., Eberwine, J., and Kim, J. (2016). Variation is function: are single cell differences functionally important? *BioEssays* 38, 172–180. doi: 10.1002/bies.201500124

Fox, R. J., Donelson, J. M., Schunter, C., Ravasi, T. C., and Gaitán-Espitia, J. D. (2019). Beyond buying time: the role of plasticity in phenotypic adaptation to rapid environmental change. *Philos. Trans. R. Soc. B* 374:20180174. doi: 10.1098/rstb.2018.0174

Furness, A. I., Lee, K., and Reznick, D. N. (2015). Adaptation in a variable environment: phenotypic plasticity and bet-hedging during egg diapause and hatching in an annual killifish. *Evolution* 69, 1461–1475. doi: 10.1111/evo.12669

Fuxjäger, L., Wanzenböck, S., Ringler, E., Wegner, K. M., Ahnelt, H., and Shama, L. N. S. (2019). Within-generation and transgenerational plasticity of mate choice in oceanic stickleback under climate change. *Philos. Trans. R. Soc. B* 374:20180183. doi: 10.1098/rstb.2018.0183

Garcia-Gonzalez, F., Yasui, Y., and Evans, J. P. (2015). Mating portfolios: bet-hedging, sexual selection and female multiple mating. *Proc. R. Soc. B* 282:20141525. doi: 10.1098/rspb.2014.1525

Gerber, N., and Kokko, H. (2018). Abandoning the ship using sex, dispersal or dormancy: multiple escape routes from challenging conditions. *Philos. Trans. R. Soc. B* 373:20170424. doi: 10.1098/rstb.2017.0424

Ghalambor, C. K., McKay, J. K., Carroll, S. P., and Reznick, D. N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Funct. Ecol.* 21, 394–407. doi: 10.1111/j.1365-2435.2007.01283.x

Gibson, G., and Wagner, G. (2000). Canalization in evolutionary genetics: a stabilizing theory? *BioEssays* 22, 372–380.

Gienapp, P., Lof, M., Reed, T. E., McNamara, J., Verhulst, S., and Visser, M. E. (2013). Predicting demographically sustainable rates of adaptation: can great tit breeding time keep pace with climate change? *Philos. Trans. R. Soc. B* 368:20120289. doi: 10.1098/rstb.2012.0289

Goossens, S., Wybouw, N., Van Leeuwen, T., and Bonte, D. (2020). The physiology of movement. *Mov. Ecol.* 8:5. doi: 10.1186/s40462-020-0192-2

Grantham, M. E., Antonio, C. J., O'Neil, B. R., Zhan, Y. X., and Brisson, J. A. (2016). A case for a joint strategy of diversified bet hedging and plasticity in the pea aphid wing polyphenism. *Biol. Lett.* 12:20160654. doi: 10.1098/rsbl.2016.0654

Green, T. R., and Ryan, C. A. (1972). Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. *Science* 175, 776–777. doi: 10.1126/science.175.4023.776

Haaland, T. R., Wright, J., and Ratikainen, I. I. (2020). Generalists versus specialists in fluctuating environments: a bet-hedging perspective. *Oikos* 129, 879–890. doi: 10.1111/oik.07109

Halkett, F., Harrington, R., Hullé, M., Kindlmann, P., Menu, F., Rispe, C., et al. (2004). Dynamics of production of sexual forms in aphids: theoretical and experimental evidence for adaptive "coin-flipping" plasticity. *Am. Nat.* 163, E112–E125. doi: 10.1086/383618

Hopper, K. R. (1999). Risk-spreading and bet-hedging in insect population biology. *Annu. Rev. Entomol.* 44, 535–560. doi: 10.1146/annurev.ento.44.1.535

IPCC (2014). *Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to The Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Geneva: IPCC.

Joschinski, J., and Bonte, D. (2020). Diapause is not selected as a bet-hedging strategy in insects: a meta-analysis of reaction norm shapes. *bioRxiv* [Preprint]. doi: 10.1101/752881

Kelly, M. (2019). Adaptation to climate change through genetic accommodation and assimilation of plastic phenotypes. *Philos. Trans. R. Soc. B* 374:20180176. doi: 10.1098/rstb.2018.0176

Kivelä, S. M., Välimäki, P., and Gotthard, K. (2016). Evolution of alternative insect life histories in stochastic seasonal environments. *Ecol. Evol.* 6, 5596–5613. doi: 10.1002/ece3.2310

Krueger, D. A., and Dodson, S. I. (1981). Embryological induction and predation ecology in *Daphnia pulex*. *Limnol. Oceanogr.* 26, 219–223. doi: 10.4319/lo.1981.26.2.0219

Kærn, M., Elston, T. C., Blake, W. J., and Collins, J. J. (2005). Stochasticity in gene expression: from theories to phenotypes. *Nat. Rev. Genet.* 6, 451–464. doi: 10.1038/nrg1615

Lane, J. E., McAdam, A. G., McFarlane, S. E., Williams, C. T., Humphries, M. M., Coltman, D. W., et al. (2018). Phenological shifts in North American red squirrels: disentangling the roles of phenotypic plasticity and microevolution. *J. Evol. Biol.* 31, 810–821. doi: 10.1111/jeb.13263

Lenton, T. M., Dakos, V., Bathiany, S., and Scheffer, M. (2017). Observed trends in the magnitude and persistence of monthly temperature variability. *Sci. Rep.* 7:5940. doi: 10.1038/s41598-017-06382-x

Li, X.-Y., Lehtonen, J., and Kokko, H. (2017). Sexual reproduction as bet-hedging [preprint]. *bioRxiv*. doi: 10.1101/103390

Luquet, E., and Tariel, J. (2016). Offspring reaction norms shaped by parental environment: interaction between within- and trans-generational plasticity of inducible defenses. *BMC Evol. Biol.* 16:209. doi: 10.1186/s12862-016-0795-9

Marcovitch, S. (1923). Plant lice and light exposure. *Science* 58, 537–538. doi: 10.1126/science.58.1513.537-a

Marshall, D. J., Bonduriansky, R., and Bussière, L. F. (2008). Offspring size variation within broods as a bet-hedging strategy in unpredictable environments. *Ecology* 89, 2506–2517. doi: 10.1890/07-0267.1

Maxwell, C. S., and Magwene, P. M. (2017). When sensing is gambling: an experimental system reveals how plasticity can generate tunable bet-hedging strategies. *Evolution* 71, 859–871. doi: 10.1111/evo.13199

Nussey, D. H., Postma, E., Gienapp, P., and Visser, M. E. (2005). Selection on heritable phenotypic plasticity in a wild bird population. *Science* 310, 304–306. doi: 10.1126/science.1117004

Oostra, V., Saastamoinen, M., Zwaan, B. J., and Wheat, C. W. (2018). Strong phenotypic plasticity limits potential for evolutionary responses to climate change. *Nat. Commun.* 9:1005. doi: 10.1038/s41467-018-03384-9

Parmesan, C., and Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature* 421:37. doi: 10.1038/nature01286

Pélisson, P.-F., Bernstein, C., François, D., Menu, F., and Venner, S. (2013). Dispersal and dormancy strategies among insect species competing for a pulsed resource. *Ecol. Entomol.* 38, 470–477. doi: 10.1111/een.12038

Perez, M. F., and Lehner, B. (2019). Intergenerational and transgenerational epigenetic inheritance in animals. *Nat. Cell Biol.* 21, 143–151. doi: 10.1038/s41556-018-0242-9

Perrin, N. (2016). Random sex determination: when developmental noise tips the sex balance. *BioEssays* 38, 1218–1226. doi: 10.1002/bies.201600093

Piao, S., Liu, Q., Chen, A., Janssens, I. A., Fu, Y., Dai, J., et al. (2019). Plant phenology and global climate change: current progresses and challenges. *Glob. Change Biol.* 25, 1922–1940. doi: 10.1111/gcb.14619

Rando, O. J., and Verstrepen, K. J. (2007). Timescales of genetic and epigenetic inheritance. *Cell* 128, 655–668. doi: 10.1016/j.cell.2007.01.023

Saikkonen, K., Taulavuori, K., Hyvönen, T., Gundel, P. E., Hamilton, C. E., Vänninen, I., et al. (2012). Climate change-driven species' range shifts filtered by photoperiodism. *Nat. Clim. Change* 2, 239–242. doi: 10.1038/nclimate1430

Scheiner, S. M., and Holt, R. D. (2012). The genetics of phenotypic plasticity. X. Variation versus uncertainty. *Ecol. Evol.* 2, 751–767. doi: 10.1002/ece3.217

Scholl, J. P., Calle, L., Miller, N., and Venable, D. L. (2020). Offspring polymorphism and bet hedging: a large-scale, phylogenetic analysis. *Ecol. Lett.* 23, 1223–1231. doi: 10.1111/ele.13522

Seger, J., and Brockmann, H. J. (1987). "What is bet-hedging?," in *Oxford Surveys in Evolutionary Biology*, eds D. J. Futuyma, and J. Antonovics (Oxford, UK: Oxford University Press), 182–211.

Simon, J.-C., Rispe, C., and Sunnucks, P. (2002). Ecology and evolution of sex in aphids. *Trends Ecol. Evol.* 17, 34–39. doi: 10.1016/S0169-5347(01)02331-X

Simons, A. M. (2011). Modes of response to environmental change and the elusive empirical evidence for bet hedging. *Proc. R. Soc. B* 278, 1601–1609. doi: 10.1098/rspb.2011.0176

Simons, A. M., and Johnston, M. O. (1997). Developmental instability as a bet-hedging strategy. *Oikos* 80, 401–406. doi: 10.2307/3546608

Simons, A. M., and Johnston, M. O. (2006). Environmental and genetic sources of diversification in the timing of seed germination: implications for the evolution of bet hedging. *Evolution* 60, 2280–2292. doi: 10.1111/j.0014-3820.2006.tb01865.x

Starrfelt, J., and Kokko, H. (2012). Bet-hedging–a triple trade-off between means, variances and correlations. *Biol. Rev.* 87, 742–755. doi: 10.1111/j.1469-185X.2012.00225.x

Stomp, M., van Dijk, M. A., van Overzee, H. M. J., Wortel, M. T., Sigon, C. A. M., Egas, M., et al. (2008). The timescale of phenotypic plasticity and its impact on competition in fluctuating environments. *Am. Nat.* 172, E169–E185. doi: 10.1086/591680

Tufto, J. (2015). Genetic evolution, plasticity, and bet-hedging as adaptive responses to temporally autocorrelated fluctuating selection: a quantitative genetic model. *Evolution* 69, 2034–2049. doi: 10.1111/evo.12716

Van Buskirk, J., and Steiner, U. K. (2009). The fitness costs of developmental canalization and plasticity. *J. Evol. Biol.* 22, 852–860. doi: 10.1111/j.1420-9101.2009.01685.x

Veening, J.-W., Smits, W. K., and Kuipers, O. P. (2008). Bistability, epigenetics, and bet-hedging in bacteria. *Annu. Rev. Microbiol.* 62, 193–210. doi: 10.1146/annurev.micro.62.081307.163002

Venable, D. L. (2007). Bet hedging in a guild of desert annuals. *Ecology* 88, 1086–1090. doi: 10.1890/06-1495

Volfson, D., Marciniak, J., Blake, W. J., Ostroff, N., Tsimring, L. S., and Hasty, J. (2006). Origins of extrinsic variability in eukaryotic gene expression. *Nature* 439, 861–864. doi: 10.1038/nature04281

Waddington, C. H. (1942). Canalization of development and the inheritance of acquired characters. *Nature* 150, 563–565. doi: 10.1038/150563a0

Woltereck, R. (1913). Weitere experimentelle untersuchungen über Artänderung, speziell über das Wesen quantitativer Artunterschiede bei Daphniden. *Mol. Gen. Genet.* 9, 146–146. doi: 10.1007/BF01876686

Woods, H. A. (2014). Mosaic physiology from developmental noise: within-organism physiological diversity as an alternative to phenotypic plasticity and phenotypic flexibility. *J. Exp. Biol.* 217, 35–45.doi: 10.1242/jeb.089698

Zhang, X.-S., and Hill, W. G. (2005). Evolution of the environmental component of the phenotypic variance: stabilizing selection in changing environments and the cost of homogeneity. *Evolution* 59, 1237–1244. doi: 10.1111/j.0014-3820.2005.tb01774.x

Zohner, C. M., Benito, B. M., Svenning, J.-C., and Renner, S. S. (2016). Day length unlikely to constrain climate-driven shifts in leaf-out times of northern woody plants. *Nat. Clim. Change* 6, 1120–1123. doi: 10.1038/nclimate3138

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Interactions Between Maternal, Paternal, Developmental, and Immediate Environmental Effects on Anti-predator Behavior of the Snail *Physa acuta*

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Transgenerational plasticity, which occurs when the environment experienced by parents changes the phenotype of offspring, is widespread in animal and plant species. Both maternal and paternal environments can underlie transgenerational plasticity, but experimental studies unraveling how their effects interact together and with the personal (both developmental and immediate) environments are still rare. Yet unraveling these interactions is fundamental to understanding how offspring integrate past and present environmental cues to produce adaptive phenotype. Using the hermaphroditic and freshwater snail *Physa acuta*, we tested how predator cues experienced by offspring, mothers and fathers interact to shape offspring anti-predator behavior. We raised a first generation of snails in the laboratory with or without chemical predator cues and realized full-factorial crosses to disentangle maternal and paternal cues. We then raised the second generation of snails with or without predator cues and assessed, when adults, their escape behavior in two immediate environments (with or without predator cues) and activity in the immediate environment without predator cues. We found that personal, maternal, and paternal predator cues interacted to shape offspring escape behavior and activity. Firstly, for escape behavior, snails integrated the cues from developmental and parental environments only when exposed to predator cues in their immediate environment, suggesting that personal immediate experience must corroborate the risky parental environment to reveal transgenerational plasticity. For activity, this same hypothesis helps explain why no clear pattern of transgenerational plasticity was revealed, as activity was only measured without predator cues in the immediate environment. Secondly, a single maternal exposure to predator cues decreased offspring escape behavior while a single paternal exposure had no effect, surprisingly demonstrating sex-specific transgenerational plasticity for a simultaneous hermaphroditic species. Thirdly, when both mother and father were exposed, paternal cues were integrated by offspring according to their own developmental environment.

The paternal exposure then mitigated the reduction in escape behavior due to the maternal exposure only when offspring developed in control condition. Overall, our study highlighted complex patterns of sex-specific transgenerational plasticity resulting from non-additive interactions between parental, developmental and immediate experiences.

Keywords: maternal effect, paternal effect, sex-specific, predator-prey interactions, cue integration, non-additive response, *Physa acuta*

INTRODUCTION

Phenotypic plasticity, i.e., change in the phenotype of an organism induced by variation in the environment, can occur within the lifetime of the organism (within-generational plasticity WGP) or across generations (transgenerational plasticity TGP). TGP can occur through its effect on parental condition: favorable or stressful environments affect parental state (e.g., body weight, reproductive performance), which in turn positively or negatively affects offspring state (state-based TGP; Donelan et al., 2020). TGP can also result from natural selection as a mechanism to pass on reliable cues to offspring about their future environment (adaptive TGP; Galloway and Etterson, 2007; Burgess and Marshall, 2014; Yin et al., 2019), allowing them to adjust their phenotype. For example, in the bryozoan *Bugula neritina*, offspring of copper-exposed mothers were more resistant to copper than those of unexposed mothers (Marshall, 2008), as maternal exposure to copper potentially indicates that the offspring environment will be copper-rich. TGP is therefore a potential mechanism of adaptation for organisms to cope with rapid environmental changes (Agrawal et al., 1999; Donelson et al., 2018). A central question is to understand how information cues and state-based constraints from parental (TGP) and personal (WGP) experiences are integrated by offspring to produce adaptive responses to environment (Stamps and Krishnan, 2014; Leimar and McNamara, 2015; Stein et al., 2018).

Research on TGP has mainly focused on effects of both parents indiscriminately or on effects of maternal environment alone. More recently, some studies have shown that paternal environment can also have a strong effect on offspring phenotype (review in Krawetz, 2005; Crean and Bonduriansky, 2014; Immler, 2018). However, the extent to which offspring attend to cues of their parents can depend on the sex of the parent leading to different maternal and paternal effects on offspring phenotype (*sex-specific TGP*; e.g., Magiafoglou and Hoffmann, 2003; Ducatez et al., 2012; Bonduriansky et al., 2016; Zuccolo et al., 2016; Emborski and Mikheyev, 2019; Gilad and Scharf, 2019; Burke et al., 2020). Sex-specific TGP may be explained by different mechanisms of cue transmission between sexes (Bell and Hellmann, 2019). Sex-specific TGP may also occur when the sexes are under different selection pressures (e.g., when sexes show sexual dimorphism, differences in reproductive strategies, dispersal or ecology) generating differences in the reliability of maternal and paternal information about the offspring future environment (Kamel et al., 2010; Bell and Hellmann, 2019; Burke et al., 2020). For example, if males and females have different ecologies, only the cue perceived by the same-sex parent can reliably predict offspring environment and offspring should then be more influenced by the same-sex parental cue (Hellmann et al., 2019; Burke et al., 2020).

The effects of maternal and paternal environments can interact with each other. TGP patterns then differ whether one parent or both parents are exposed to environmental variation. This interaction may be even more complicated as offspring also integrate cues from their own personal environments. Personal cues can come from offspring's past experiences during ontogeny or from their immediate environment. Integrating multiple cues (maternal, paternal and personal) may allow to fine-tune the offspring phenotype according to past and present environmental information. However, it is still not clear how offspring integrate these different sources of information, sometimes consistent or conflicting, and prioritize them. Effects of cues may simply be additive: an increasing number of cues in agreement increases linearly with adaptive phenotype (additive effects; e.g., Akkerman et al., 2016; Zizzari et al., 2016). Effects of cues may also interact and result in non-additive effects (e.g., Galloway, 2001; Hellmann et al., 2019). For example, offspring may respond similarly whether they receive one or several cues in agreement (back-up hypothesis; Bell and Hellmann, 2019). Offspring may also respond only when their personal cues corroborate parental cues or only when both parental cues are consistent (threshold hypothesis; Bell and Hellmann, 2019). Thus, understanding how the effects of personal, maternal and paternal environments interact is necessary to understand variations in TGP patterns and the possible implication of parental sex in its evolution.

In this laboratory study, we investigated how effects of maternal, paternal and personal (both developmental and immediate) exposures to cues of predator presence interact to shape anti-predator behaviors. Many prey engage in anti-predatory behaviors when they or their parents detect predator cues (predator-induced WGP: Lima and Dill, 1990; predator-induced TGP: review in Tariel et al., 2020). We used the freshwater snail *Physa acuta* as our model system. *Physa* species are known for their anti-predator behavior: they escape by crawling-out the water or seek refuge after detecting crayfish or fish odors (Alexander and Covich, 1991; DeWitt et al., 1999; Turner et al., 1999). One study has also shown a reduction of activity after detection of crayfish odors (Sih and McCarthy, 2002). We have already shown predator-induced WGP and TGP on escape behavior of *P. acuta* (Luquet and Tariel, 2016; Tariel et al., 2020), while another study detected only WGP and no TGP (Beaty et al., 2016). However, none of these studies have yet disentangled the potential interactions between immediate, developmental, maternal and paternal environments. We raised

a parental generation of snails with or without chemical predator cues (non-lethal predators) and realized full-factorial crosses. We then raised the offspring generation with or without predator cues. At the adult stage, we measured two anti-predator behaviors: (1) escape behavior in two immediate environments (with or without predator cues) and (2) activity without predator cues in the immediate environment. *P. acuta* is a simultaneous hermaphrodite with internal fertilization and does not benefit from any parental care. These reproductive characteristics imply that the mother (egg donor) and father (sperm donor) have the same ecology, gamete dispersal and no sexual dimorphism: predator cues perceived by the mother and father should carry the same information about future predator presence and trigger the same response on offspring anti-predator behavior; hence, we do not expect sex-specific TGP (Bell and Hellmann, 2019; Burke et al., 2020). We can then more easily study how personal (immediate and developmental), maternal and paternal cues interact to influence offspring anti-predator behaviors.

MATERIALS AND METHODS

Experimental Design

Wild adult *Physa acuta* snails were collected in February 2017 in a lentic backwater of the Rhône River in Lyon, France (N 45°48′06″ E 04°55′33″). *P. acuta* is a globally invasive freshwater snail from North America (Lydeard et al., 2016). These snails constituted the F0 generation (see **Figure 1** for number of individuals; see **Appendix 1** for the schematic experimental design). In the laboratory, F0 snails were pooled in a 10 L plastic box filled with dechlorinated tap water (control water hereafter) and interbreed overnight. Then, they were isolated in 80 mL plastic boxes (these rearing boxes were used until the end of the experiment; 4.5 × 6 cm) filled with control water to ensure that a box contained the progeny of only one F0 snail (i.e., one F1 family per box). They laid eggs during 24 h, which was enough for most snails to lay an egg mass, and then were removed. F1 eggs developed until hatching at 25°C (ca. 7 days). After hatching and until the end of snail development, they were reared in a room at 25°C with a 12 h/12 h photoperiod. Snails were fed *ad libitum* with boiled and mixed lettuce. The water and food were renewed twice a week. Siblings of each F1 family developed together for 10 days in control water without being manipulated (*P. acuta* newborns are very small and easily damaged). Then, siblings of each F1 family were split into two developmental environments: six siblings remained in control (C) water and six siblings moved to predator-cue (P) water (**Figure 1**). F1 families with less than 12 siblings were discarded (see **Figure 1** for number of families). After 7 days, F1 snails were isolated in the same environment (control or predator-cue water) and developed individually until 31 days old to reach a size large enough to be sexually mature (see **Figure 1** for number of individuals). The predator-cue water was obtained by (1) mixing the rearing water of six *Orconectes limosus* crayfish (reared individually in 4 L box of dechlorinated tap water) and (2) "infusing" several smashed *P. acuta* adult snails (one snail for every 4 L) for 1 h before using the predator-cue water. These smashed snails were then used as crayfish food.

To generate the F2 generation, we performed three types of pairs with F1 snails (each partner in a pair generated a progeny as *P. acuta* is a hermaphrodite; **Figure 1**):

(1) the two partners coming from control environment, to generate combination of maternal C × paternal C environments;
(2) the two partners coming from predator-cue environment, to generate combination of maternal P × paternal P environments;
(3) the two partners coming from different environments, to generate combinations of maternal C × paternal P environments and maternal P × paternal C environments.

We paired partners from different families, and we were careful to associate the same families in the three types of pairs. To keep the partner identity during copulation, we painted shells (Henry and Jarne, 2007). We put the two partners into a rearing box for 24 h to copulate (*P. acuta* prefers outcross; Tsitrone et al., 2003). We then submitted snails of the F2 generation to the two treatments (control or predator-cue) following the same protocol as for the F1 generation (see **Figure 1** for number of individuals). As F2 snails developed slower, they developed until 61 days old to reach a size large enough for their weight to be accurately measured.

Finally, our full factorial design resulted in four combinations of maternal and paternal environments (CxC, PxC, CxP, PxP) and in two F2 offspring developmental environments (C, P) (**Figure 1**).

Assessing Anti-predator Behaviors

We assessed two anti-predator behaviors: escape behavior by the time taken to crawl-out the water (sec) and activity by the total covered distance (cm). These two behaviors were assessed on two different subsamples of F2 snails (**Figure 1**). The sub-samples were composed of as many different F2 families as possible.

For each trial of escape behavior, the snail first acclimated in a chamber at the center of a behavioral arena (4.5 × 6 cm box with 2.3 cm water and 0.7 cm polystyrene at the bottom). A minute later, we opened the acclimation chamber and recorded time taken by the snail to crawl to the water surface with JWatcher (Blumstein and Daniel, 2007). After 5 min, we put back the snail in its rearing box for at least 1 h before the next trial and we changed arena water. For each snail, the escape behavior trial was repeated two times in each immediate environment (two times in control water first and two times in predator-cue water after).

For each trial of activity, the snail first acclimated 1 min in the entire behavioral arena (11.5 × 17.5 cm box with 0.6 cm water). We then recorded the snail position every 30 s on a grid (0.5 cm tiles) drawn at the bottom of the arena. After 5 min, we put back the snail in its rearing box for at least 1 h before the next trial and we changed arena water. The activity trial was repeated two times only in control water. As snails escape (crawl-out the water) in predator-cue water, the activity could not be assessed in this immediate environment.

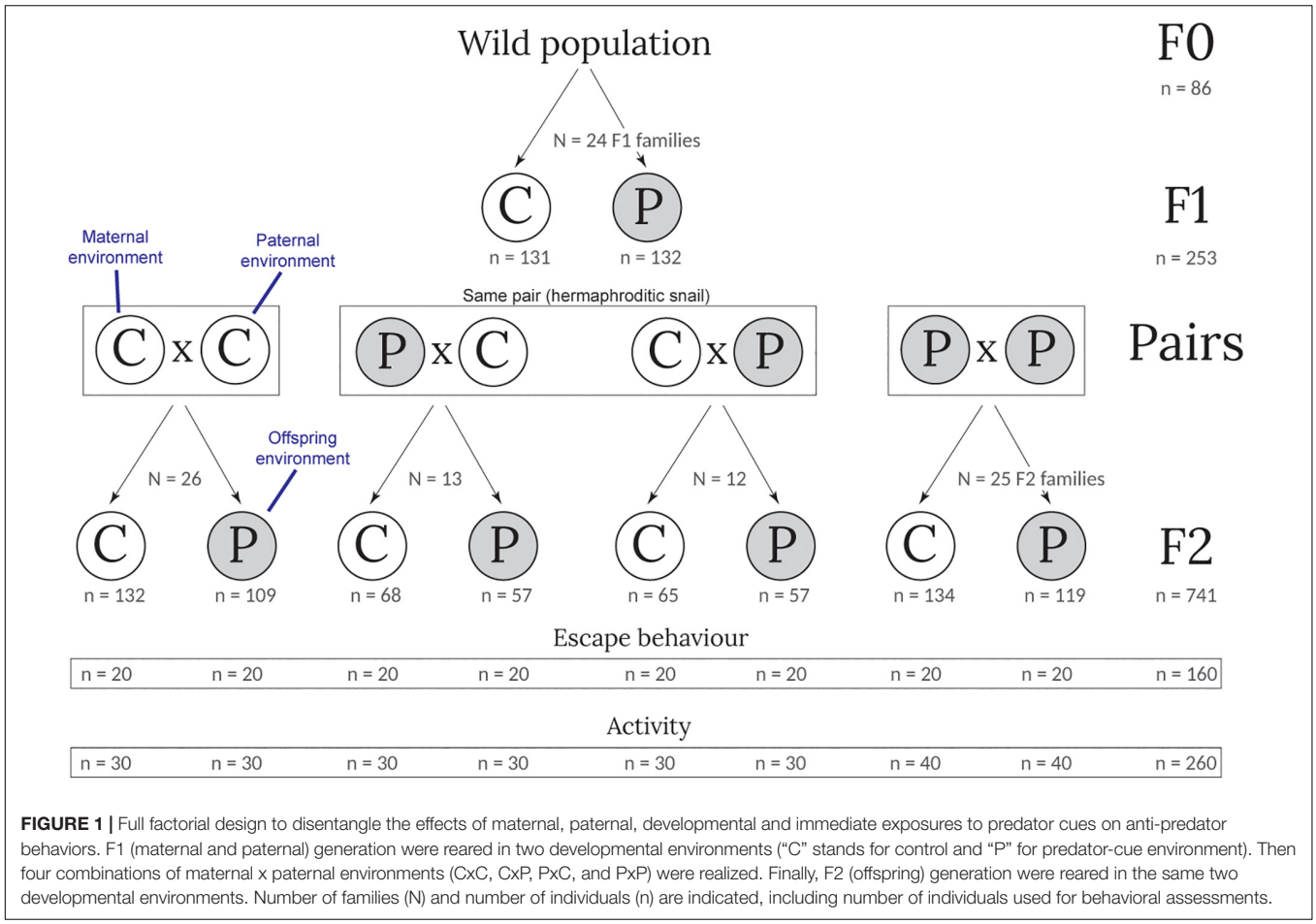

FIGURE 1 | Full factorial design to disentangle the effects of maternal, paternal, developmental and immediate exposures to predator cues on anti-predator behaviors. F1 (maternal and paternal) generation were reared in two developmental environments ("C" stands for control and "P" for predator-cue environment). Then four combinations of maternal x paternal environments (CxC, CxP, PxC, and PxP) were realized. Finally, F2 (offspring) generation were reared in the same two developmental environments. Number of families (N) and number of individuals (n) are indicated, including number of individuals used for behavioral assessments.

After assessing behavior, we gently dried snails with a paper towel and measured total wet mass (body and shell) with an electronic scale at the nearest 0.0001 g.

Statistical Analysis

We tested with two separate linear mixed models (LMMs) whether maternal, paternal and developmental environments influenced F2 snails' escape behavior and activity. For the escape behavior, a LMM was used instead of a survival model as just a few trials were censored (censoring means that the snail did not crawl-out the water within the five min trial; 32 trials out of 640 trials). The time to crawl-out was transformed with a log10 transformation to achieve normality. We also multiplied by −1 the time to crawl-out to better reflect escape behavior, as a short time to crawl-out is associated with high escape behavior. The fixed effects of the two LMMs included maternal (E1m), paternal (E1p) and developmental (E2) environments and all interactions. For the LMM of escape behavior, we added the immediate (I) environment and all interactions. For all LMMs, we also added the scaled snail mass to control for size. We reduced the number of fixed effects using the model selection explained in Zuur et al. (2009). The random effect of the two LMMs included individual identity to control for intrinsic differences in behavior between individuals. We fitted LMMs with restricted maximum likelihood estimation and Kenward and Roger's approximation for degrees of freedom using the lmer() function (Bates et al., 2015). Type 2 F-tests were used for significance of fixed effects with the package lmerTest (Kuznetsova et al., 2017). *Post hoc* comparisons were realized with the package emmeans (Lenth, 2019) to compare the "control" treatment (both parents not exposed: CxC) with the other treatments (only exposed mothers: PxC; only exposed fathers: CxP; and both parents exposed: PxP). These *post hoc* comparisons are reported in **Appendix 2**. All statistical analyses were performed with R 3.6.0 (R Development Core Team, 2019).

RESULTS

Interactions Between Maternal, Paternal, and Personal Environments on Offspring Escape Behavior

Maternal (E1m), paternal (E1p), developmental (E2) and immediate (I) environments all influenced escape behavior and their effects interacted with each other (significant E1m × E1p × E2 interaction and E1m × E1p × I interaction in **Table 1** and **Figure 2**). In the immediate environment without

TABLE 1 | Results on the linear mixed model on escape behavior.

Fixed effects	Estimate (SE)	NumDF, DenDF	*F*	*P*
Mass	0.02 (0.011)	1, 151	3.40	0.067
Maternal treatment (E1m)	−0.04 (0.047)	1, 151	8.22	**0.005**
Paternal treatment (E1p)	0.06 (0.047)	1, 151	5.64	**0.019**
Developmental treatment (E2)	−0.03 (0.046)	1, 151	4.40	**0.038**
Immediate treatment (I)	0.25 (0.032)	1, 475	36.77	**<0.001**
E1m × E1p	0.06 (0.067)	1, 151	2.37	0.126
E1m × E2	0.10 (0.061)	1, 151	0.14	0.709
E1m × I	0.03 (0.061)	1, 151	1.70	0.195
E1p × E2	−0.21 (0.041)	1, 475	19.40	**<0.001**
E1p × I	−0.12 (0.041)	1, 475	1.04	0.308
E2 × I	−0.08 (0.029)	1, 475	7.82	**0.005**
E1m × E1p × E2	−0.17 (0.086)	1, 151	4.04	**0.046**
E1m × E1p × I	0.18 (0.058)	1, 475	9.29	**0.002**
Random effect	**Variance**	**df**	χ^2	***P***
Individual	0.010	1	37.15	**<0.001**
Residual	0.033			

Bold p-values indicate significant p-value ($P < 0.05$).

predator cues, neither maternal nor paternal environments influenced escape behavior (**Figure 2A** and **Appendix 2**). In the immediate environment with predator cues, both maternal and paternal environments influenced escape behavior. The single maternal exposure to predator cues (PxC) resulted in a 49 s (61%) slower escape than unexposed parents (CxC; black solid arrows on **Figure 2B**) while the single paternal exposure (CxP) did not influence escape behavior (**Figure 2B** and **Appendix 2**). The combination of maternal and paternal exposures (PxP) resulted in a similar time to escape than unexposed parents (CxC) when offspring developed without predator cues (**Figure 2Bi** and **Appendix 2**). Conversely, the combination of maternal and paternal exposures (PxP) resulted in a 28 s (31%) slower escape than unexposed parents (CxC) when offspring developed with predator cues (**Figure 2Bii** and **Appendix 2**).

Differences in escape behavior among snails were consistent across the different behavioral trials (significant individual random effect in **Table 1**).

Interactions Between Maternal, Paternal and Personal Environments on Offspring Activity

Maternal (E1m), paternal (E1p) and developmental (E2) environments all influenced activity and their effects interacted with each other (significant E1m × E1p × E2 interaction in **Table 2** and **Figure 3**). However, *post hoc* comparisons did not reveal significant differences between the activity of offspring from unexposed parents (CxC) to the activity of offspring from exposed mothers, exposed fathers or both exposed parents (PxC, CxP, or PxP).

Differences in activity among snails were consistent across the different behavioral trials (significant individual random effect in **Table 2**).

DISCUSSION

We know little about how offspring integrate cues from the maternal, paternal, developmental and immediate environments (Stein et al., 2018). Recent theory showed that the relative weight given to a cue depends on its accuracy as a predictor of selective conditions in the future (Leimar and McNamara, 2015). In the context of maternal and paternal cue integration, both additive (Akkerman et al., 2016) and non-additive (e.g., Galloway, 2001; Valtonen et al., 2012; Guillaume et al., 2016) TGP patterns have been observed. Our results show that all past (maternal, parental, and developmental) and immediate environmental experiences had an influence on the offspring anti-predator behaviors resulting in a complex non-additive TGP patterns.

Immediate Environment as the Most Accurate Information

The maternal, paternal, and developmental environments had an influence on escape behavior but only in the immediate context of predation risk. This means that snails integrated the cues from parental and developmental environments only in the light of the immediate environment (**Figure 2B**). The immediate environment being the most accurate information about the predation risk, the snails did not exhibit differences in escape behavior when the immediate environment was safe (without predator cues), even if they or their parents were exposed to predator cues. Indeed, the maternal exposure decreased the escape behavior of offspring only in the immediate predation environment whatever the offspring developmental environment (**Figure 2B**). In the immediate safe environment (**Figure 2A**), this negative maternal effect is likely masked by an overall absence of behavioral response. Indeed, the time to crawl-out in immediate safe environment was ca. 150 s for all snails (**Figure 2A**) while in the immediate predation environment

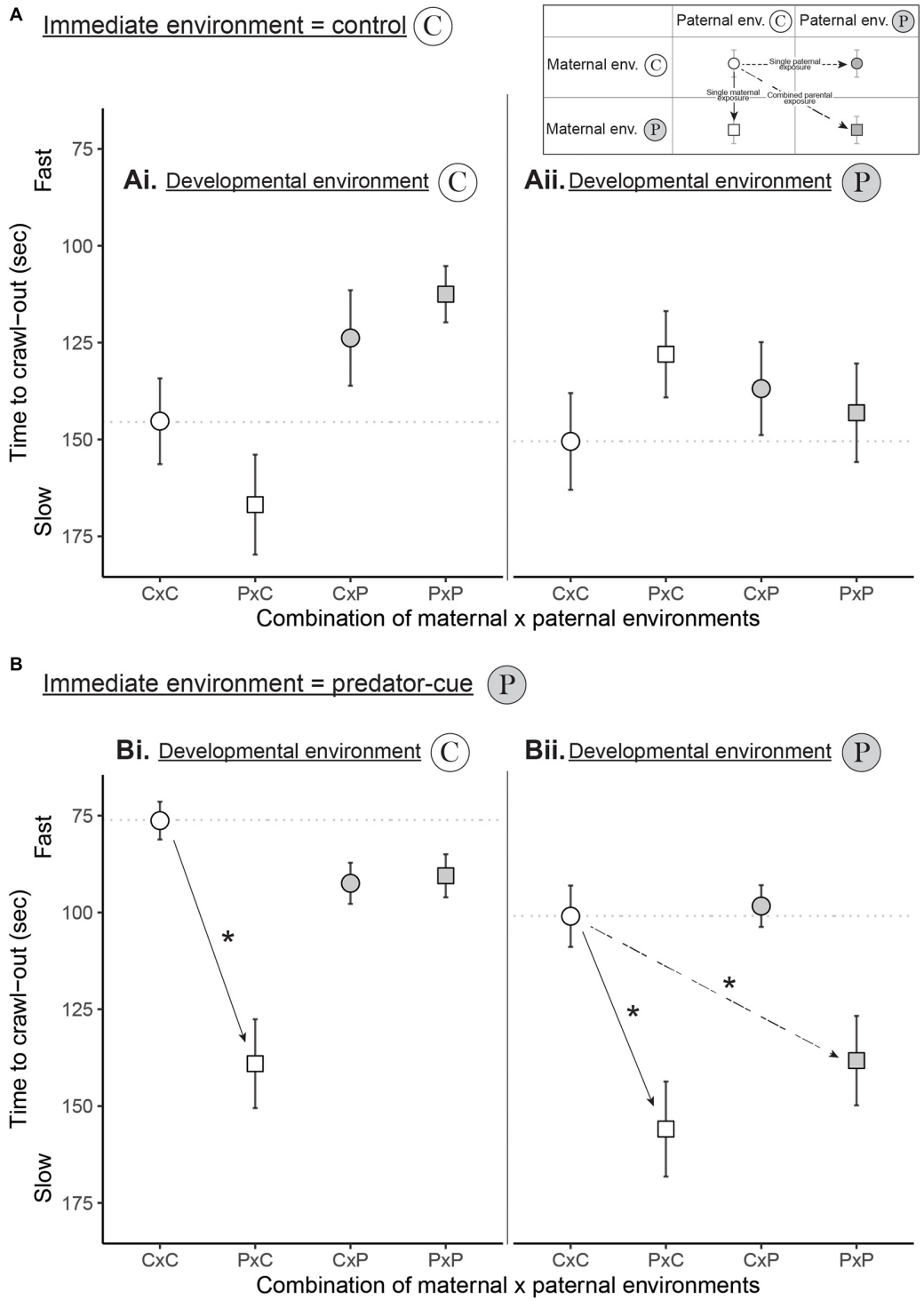

FIGURE 2 | How maternal, paternal, and developmental exposures to predator cues influenced escape behavior (time to crawl-out the water in seconds) in the **(A)** control and **(B)** predator-cue immediate environments. "C" stands for control environment and "P" stands for predator-cue environment. On each graph, the "C" developmental environment is shown on the left panel (Ai, Bi) and the "P" development environment is shown on the right panel (Aii, Bii). The *x*-axis represents the four combinations of maternal x paternal environments (CxC, PxC, CxP, and PxP). The horizontal dotted line is located at the mean of CxC to facilitate the comparisons with PxC, CxP, and PxP (see **Appendix 2** for *post hoc* comparisons). Dots are for snails from non-exposed mothers while squares are for snails from exposed mothers. White shapes are for snails from non-exposed fathers while gray shapes are for snails from exposed fathers. To see the effect of single maternal exposure, single paternal exposure or combined parental exposures, follow the legend on the top right corner. Black arrows indicate a significant effect (**Appendix 2**). Points are mean ± SE.

TABLE 2 | Results on the linear mixed model on activity.

Fixed effects	Estimate (SE)	NumDF, DenDF	*F*	*P*
Mass	1.79 (0.617)	1, 251	8.41	**0.004**
Maternal treatment (E1m)	3.23 (2.468)	1, 251	0.91	0.340
Paternal treatment (E1p)	5.24 (2.471)	1, 251	4.88	**0.028**
Developmental treatment (E2)	1.97 (2.467)	1, 251	0.00	0.960
E1m × E1p	−5.97 (3.388)	1, 251	0.19	0.664
E1m × E2	−5.30 (3.496)	1, 251	0.83	0.364
E1p × E2	−6.26 (3.495)	1, 251	0.25	0.616
E1m × E1p × E2	13.98 (4.789)	1, 251	8.52	**0.004**
Random effect	**Variance**	**df**	χ^2	***P***
Individual	31.07	1	9.37	**<0.001**
Residual	120.48			

Bold p-values indicate significant p-value ($P < 0.05$).

(**Figure 2B**), only the snails from exposed mothers took 150 s to escape, the others snails escaping faster (ca. 90 s). This negative maternal effect is likely revealed only when offspring must express behavioral defenses against predators (faster escape in immediate predation environment).

For activity of offspring, we found consistently that maternal, paternal, and developmental environments interacted. However, we did not identify significant patterns resulting from this triple interaction. As for escape behavior, where we did not observe significant differences among snails in the immediate environment without predator cues, the immediate environment is likely the first to be integrated by offspring. In absence of immediate predator cues, offspring did not alter their activity according to parental and developmental environments while in the presence of predator cues, activity cannot be measured as all snails exhibited an escape behavior.

Sex-Specific Transgenerational Plasticity: A Negative Maternal Effect

In our predator-prey system, we did not expect sex-specific TGP as *P. acuta* is a simultaneous hermaphrodite with an internal fertilization and does not provide any parental care. The mating partners therefore have the same morphology, behavior, ecology, and gamete dispersal; they just differ in the reproduction function used at the time of mating (female: eggs and mucus; male: sperm and seminal fluid). Hence, they experience the same predation pressures and may have the same phenotypic optima in response to the current predation risk. This suggests that (1) both sexes would exert similar effects on offspring traits and (2) offspring would similarly benefit to process the maternal or paternal cues (Kamel et al., 2010; Bell and Hellmann, 2019). Surprisingly, we found sex-specific TGP on escape behavior in the immediate context of predation risk, meaning that female and male functions have distinct effects on this trait depending on the immediate environment. For activity (only measured without predator cues), no distinct effects of maternal or paternal environments were found. Investigations of such sex-specific TGP in simultaneous hermaphrodites is restricted to plant species. However, in plants, huge differences in gamete and seed dispersals between male and female functions may promote sex-specific TGP (Galloway, 2001; but see Akkerman et al., 2016). In animals, evidence of sex-specific TGP often used species with sex-specific selection pressures or species providing uniparental care (e.g., Shama et al., 2014; Emborski and Mikheyev, 2019; Lehto and Tinghitella, 2020). Our results suggest that sex-specific TGP may be driven by other factors than sex-specific selection pressures and uniparental care.

Firstly, sex-specific TGP in *P. acuta* may arise from differences in inheritance mechanisms between female and male functions (Akkerman et al., 2016; Guillaume et al., 2016; Bell and Hellmann, 2019). Female function has a narrow relationship with the subsequent generation and can add informative molecules (e.g., hormones, non-coding RNA) or nutrients in eggs during the production of oocytes or during the offspring very early development (after fertilization and before laying). By contrast, male function can only influence offspring via sperm cells and seminal fluid and therefore has a limited number of molecular pathways to transmit information to the subsequent generation (e.g., epigenetic mechanisms). Although we do not know the specific inheritance mechanisms acting in our study, we can suppose that female and male functions transmit environmental cues via different bearers of information that would explain this sex-specific TGP for escape behavior. For example, in yellow monkeyflower (*Mimulus guttatus*), Akkerman et al. (2016) have shown that demethylation erased the effect of maternal but not paternal environment, suggesting that only maternal information is transmitted via DNA methylation.

Secondly, sex-specific TGP on escape behavior may result from stronger effects of stress on female function than on male function. The single maternal exposure to predator cues induced a decrease in escape behavior (offspring took longer to crawl-out the water) while the single paternal exposure to predator cues did not influence the escape behavior. These different effects between female and male parental functions on offspring behavior may result from differences in reproductive investment. As in numerous organisms, the female function is likely more costly than the male one in *P. acuta*, although

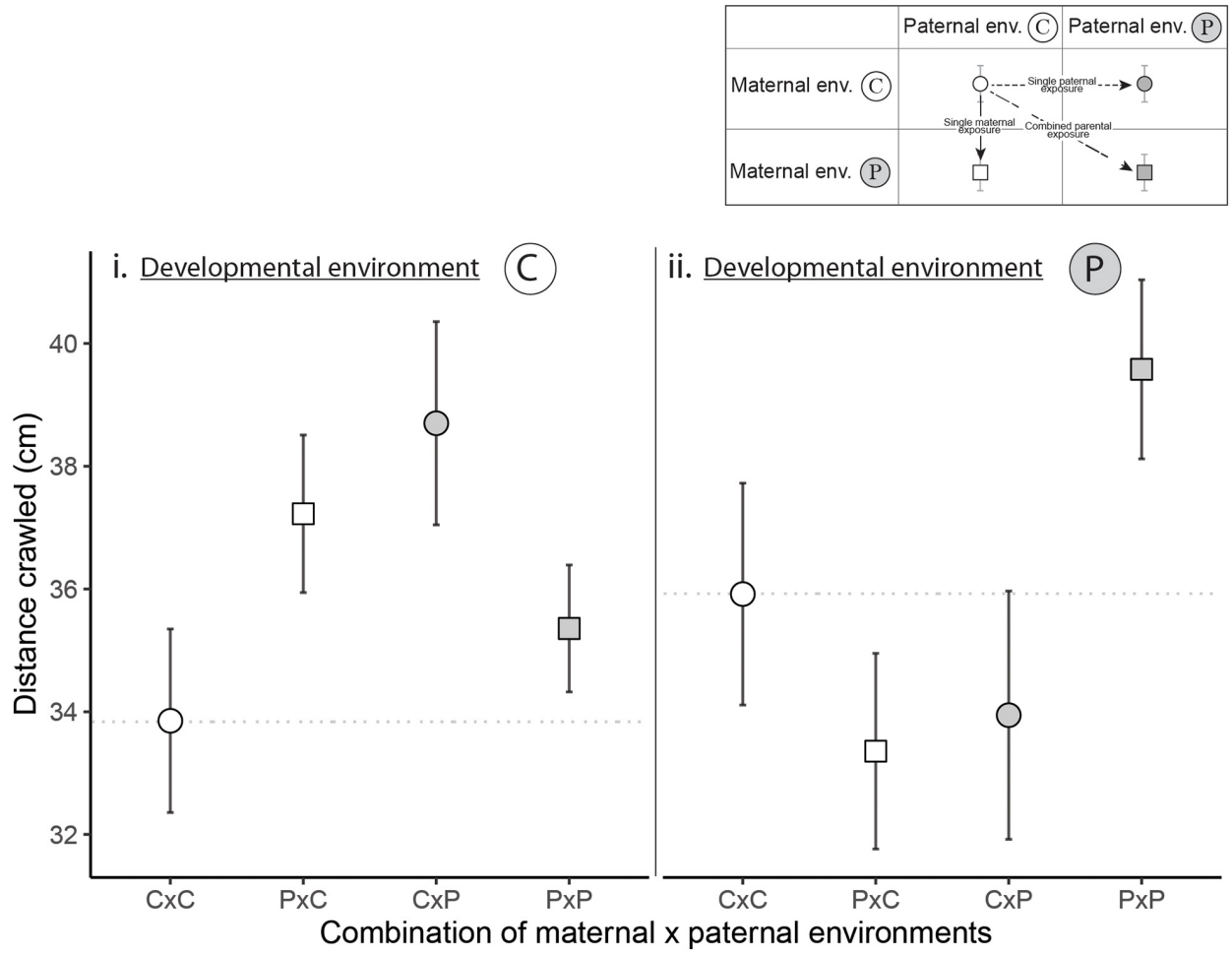

FIGURE 3 | How maternal, paternal and developmental exposures to predator cues influenced activity (covered distance in centimeters) in control immediate environment. "C" stands for control environment and "P" for predator-cue environment. The "C" developmental environment is shown on the left panel (i) and the "P" developmental environment is shown on the right panel (ii). The *x*-axis represents the four combinations of maternal x paternal environments (CxC, PxC, CxP, and PxP). The horizontal dotted line is located at the mean of CxC to facilitate the comparisons with PxC, CxP, and PxP (see **Appendix 2** for *post hoc* comparisons). Dots are for snails from non-exposed mothers while squares are for snails from exposed mothers. White shapes are for snails from non-exposed fathers while gray shapes are for snails from exposed fathers. To see the effect of single maternal exposure, single paternal exposure or combined parental exposures, follow the legend on the top right corner. Points are mean ± SE.

it is difficult to evaluate it in a simultaneous hermaphrodite. Therefore, stressful environments (such as with predation risk) that negatively influence the snail's state (DeWitt, 1998) are more likely to be passed on by the female function than by the male function. This hypothesis is corroborated by the decrease in escape behavior induced by the maternal exposure. Although in this study we did not test offspring survival in the face of a real predator, our results (**Appendix 3**) and other studies (e.g., DeWitt et al., 1999; Beaty et al., 2016) have shown that a faster escape behavior of snails exposed to predator-cues in their immediate environment is adaptive. Consequently, the decrease in snail escape behavior induced by maternal exposure may have deleterious fitness effects on offspring in response to a predator. The negative direction of the maternal effect therefore suggests that the maternal effect is a state-based effect rather than an anticipatory one (see also Coslovsky and Richner, 2011; McGhee et al., 2012). Finally, the combination of stressful environments and difference in reproductive investment between sexes may be sufficient to generate a state-based TGP that is sex-specific.

A Complex Pattern of Paternal Effect on Escape Behavior: Combination of Maternal and Developmental Effects

In the immediate predation environment (**Figure 2B**), the effect of paternal cues depended on whether they were integrated alone or in combination with maternal and developmental cues (see also Galloway, 2001). This results in a complex interaction between paternal, maternal and offspring environments that we can dissect as follows: (1) The single paternal exposure never affected the offspring escape behavior whatever the offspring

developmental environment; (2) When both mother and father were exposed, paternal cues were integrated by offspring according to their own developmental environment. In a developmental environment without predator cues (**Figure 2Bi**), the paternal exposure mitigated the reduction in escape behavior due to the maternal exposure. Consequently, offspring from both exposed parents escaped as fast as offspring from unexposed parents. This mitigation suggests that offspring can process the cues from exposed fathers to buffer the maternal state-based effect, which we can interpret as an information-based TGP of the paternal cues. Similar results have been observed in sticklebacks (*Gasterosteus aculeatus*) where the negative effect of paternal exposure on offspring survival in the face of a real predator was mitigated by maternal exposure (Hellmann et al., 2019). In this species, as males are the sole providers of paternal care, a paternal exposure to predators leads to negative stress-mediated effects. In a developmental environment with predator cues (**Figure 2Bii**), the paternal exposure combined with the maternal one did not compensate the negative maternal effect resulting in a slow behavioral response of snails, similar as observed from exposed mother alone. The exposure to predator cues during offspring development might influence the offspring state (DeWitt, 1998) that might be then no longer able to process the paternal cues to mitigate the negative effect of their exposed mothers.

The non-additive patterns we observed here do not fit with the responses already observed in the literature (threshold hypothesis: Stein et al., 2018; Bell and Hellmann, 2019; Lehto and Tinghitella, 2019, 2020; back-up hypothesis: Bell and Hellmann, 2019; Lehto and Tinghitella, 2019; bayesien hypothesis: Stamps and Krishnan, 2014). We showed that the non-additive responses of offspring for escape behavior might result from a hierarchical integration of cues (i.e., information-based) constrained by state-based effects. Immediate and paternal exposures to predator cues might led to information-based effects on the escape behavior of offspring while developmental and maternal effects might have negative state-based effects. Hence, offspring might first integrate the cues from their immediate environment (the most accurate predictor of predation risk) before to integrate then the cues from their fathers (mitigation of the negative maternal effect) if their state is not constrained by their own developmental environment (without predator cues).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SP and ÉL designed the project and carried out the experiments. JT carried out the data analysis and drafted the manuscript. All authors strongly revised the manuscript and approved the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.591074/full#supplementary-material

REFERENCES

Agrawal, A. A., Laforsch, C., and Tollrian, R. (1999). Transgenerational induction of defences in animals and plants. *Nature* 401, 60–63. doi: 10.1038/43425

Akkerman, K. C., Sattarin, A., Kelly, J. K., and Scoville, A. G. (2016). Transgenerational plasticity is sex-dependent and persistent in yellow monkeyflower (*Mimulus guttatus*). *Environ. Epigenet.* 2:dvw003. doi: 10.1093/eep/dvw003

Alexander, J. E., and Covich, A. P. (1991). Predator avoidance by the freshwater snail *Physella virgata* in response to the crayfish *Procambarus simulans*. *Oecologia* 87, 435–442. doi: 10.1007/BF00634603

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–48. doi: 10.18637/jss.v067.i01

Beaty, L. E., Wormington, J. D., Kensinger, B. J., Bayley, K. N., Goeppner, S. R., Gustafson, K. D., et al. (2016). Shaped by the past, acting in the present: transgenerational plasticity of anti-predatory traits. *Oikos* 125, 1570–1576. doi: 10.1111/oik.03114

Bell, A. M., and Hellmann, J. (2019). An integrative framework for understanding the mechanisms and multigenerational consequences of transgenerational plasticity. *Annu. Rev. Ecol. Evol. Syst.* 50, 97–118. doi: 10.1146/annurev-ecolsys-110218-024613

Blumstein, D. T., and Daniel, J. C. (2007). *Quantifying Behavior the JWatcher Way*. Sunderland, MA: Sinauer Associates.

Bonduriansky, R., Runagall-McNaull, A., and Crean, A. J. (2016). The nutritional geometry of parental effects: maternal and paternal macronutrient consumption and offspring phenotype in a neriid fly. *Funct. Ecol.* 30, 1675–1686. doi: 10.1111/1365-2435.12643

Burgess, S. C., and Marshall, D. J. (2014). Adaptive parental effects: the importance of estimating environmental predictability and offspring fitness appropriately. *Oikos* 123, 769–776. doi: 10.1111/oik.01235

Burke, N. W., Nakagawa, S., and Bonduriansky, R. (2020). Sexual conflict mediated by ecological sex differences can generate diverse patterns of transgenerational plasticity. *bioRxiv* [Preprint]. doi: 10.1101/846287 bioRxiv: 846287,

Coslovsky, M., and Richner, H. (2011). Predation risk affects offspring growth via maternal effects: maternal effects due to predation risk. *Funct. Ecol.* 25, 878–888. doi: 10.1111/j.1365-2435.2011.01834.x

Crean, A. J., and Bonduriansky, R. (2014). What is a paternal effect? *Trends Ecol. Evol.* 29, 554–559. doi: 10.1016/j.tree.2014.07.009

DeWitt, T. J. (1998). Costs and limits of phenotypic plasticity: tests with predator-induced morphology and life history in a freshwater snail. *J. Evol. Biol.* 11, 465–480. doi: 10.1046/j.1420-9101.1998.11040465.x

DeWitt, T. J., Sih, A., and Hucko, J. A. (1999). Trait compensation and cospecialization in a freshwater snail: size, shape and antipredator behaviour. *Anim. Behav.* 58, 397–407. doi: 10.1006/anbe.1999.1158

Donelan, S. C., Hellmann, J. K., Bell, A. M., Luttbeg, B., Sheriff, M. J., and Sih, A. (2020). Transgenerational plasticity in human-altered environments. *Trends Ecol. Evol.* 35, 115–124. doi: 10.1016/j.tree.2019.09.003

Donelson, J. M., Salinas, S., Munday, P. L., and Shama, L. N. S. (2018). Transgenerational plasticity and climate change experiments: where do we go from here? *Glob. Change Biol.* 24, 13–34. doi: 10.1111/gcb.13903

Ducatez, S., Baguette, M., Stevens, V. M., Legrand, D., and Fréville, H. (2012). Complex interactions between paternal and maternal effects: parental experience and age at reproduction affect fecundity and offspring performance in a butterfly. *Evolution* 66, 3558–3569. doi: 10.1111/j.1558-5646.2012.01704.x

Emborski, C., and Mikheyev, A. S. (2019). Ancestral diet transgenerationally influences offspring in a parent-of-origin and sex-specific manner. *Philos. Trans. R. Soc. B Biol. Sci.* 374:20180181. doi: 10.1098/rstb.2018.0181

Galloway, L. F. (2001). The effect of maternal and paternal environments on seed characters in the herbaceous plant *Campanula americana* (Campanulaceae). *Am. J. Bot.* 88, 832–840. doi: 10.2307/2657035

Galloway, L. F., and Etterson, J. R. (2007). Transgenerational plasticity is adaptive in the wild. *Science* 318, 1134–1136. doi: 10.1126/science.1148766

Gilad, T., and Scharf, I. (2019). Separation between maternal and paternal effects on offspring following exposure of adult red flour beetles to two stressors. *Ecol. Entomol.* 44, 494–501. doi: 10.1111/een.12726

Guillaume, A. S., Monro, K., and Marshall, D. J. (2016). Transgenerational plasticity and environmental stress: do paternal effects act as a conduit or a buffer? *Funct. Ecol.* 30, 1175–1184. doi: 10.1111/1365-2435.12604

Hellmann, J. K., Bukhari, S. A., Deno, J., and Bell, A. M. (2019). Sex-specific transgenerational plasticity in threespined sticklebacks. *bioRxiv* [Preprint]. doi: 10.1101/763862 bioRxiv: 763862,

Henry, P.-Y., and Jarne, P. (2007). Marking hard-shelled gastropods: tag loss, impact on life-history traits, and perspectives in biology. *Invertebr. Biol.* 126, 138–153. doi: 10.1111/j.1744-7410.2007.00084.x

Immler, S. (2018). The sperm factor: paternal impact beyond genes. *Heredity* 121, 239–247. doi: 10.1038/s41437-018-0111-0

Kamel, S. J., Grosberg, R. K., and Marshall, D. J. (2010). Family conflicts in the sea. *Trends Ecol. Evol.* 25, 442–449. doi: 10.1016/j.tree.2010.05.008

Krawetz, S. A. (2005). Paternal contribution: new insights and future challenges. *Nat. Rev. Genet.* 6, 633–642. doi: 10.1038/nrg1654

Kuznetsova, A., Brockhoff, P. B., and Christensen, R. H. B. (2017). lmertest package: tests in linear mixed effects models. *J. Stat. Softw.* 82, 1–26. doi: 10.18637/jss.v082.i13

Lehto, W. R., and Tinghitella, R. M. (2019). Joint maternal and paternal stress increases the cortisol in their daughters' eggs. *Evol. Ecol. Res.* 20, 133–144.

Lehto, W. R., and Tinghitella, R. M. (2020). Predator-induced maternal and paternal effects independently alter sexual selection. *Evolution* 74, 404–418. doi: 10.1111/evo.13906

Leimar, O., and McNamara, J. M. (2015). The evolution of transgenerational integration of information in heterogeneous environments. *Am. Nat.* 185, E55–E69. doi: 10.1086/679575

Lenth, R. (2019). *emmeans: Estimated Marginal Means, aka Least-Squares Means*. Available online at: https://CRAN.R-project.org/package=emmeans.

Lima, S. L., and Dill, L. M. (1990). Behavioral decisions made under the risk of predation: a review and prospectus. *Can. J. Zool.* 68, 619–640. doi: 10.1139/z90-092

Luquet, E., and Tariel, J. (2016). Offspring reaction norms shaped by parental environment: interaction between within- and trans-generational plasticity of inducible defenses. *BMC Evol. Biol.* 16:209. doi: 10.1186/s12862-016-0795-9

Lydeard, C., Campbell, D., and Golz, M. (2016). *Physa acuta* Draparnaud, 1805 should be treated as a native of North America. not Europe. *Malacologia* 59, 347–350. doi: 10.4002/040.059.0213

Magiafoglou, A., and Hoffmann, A. A. (2003). Cross-generation effects due to cold exposure in *Drosophila serrata*. *Funct. Ecol.* 17, 664–672. doi: 10.1046/j.1365-2435.2003.00774.x

Marshall, D. J. (2008). Transgenerational plasticity in the sea: context-dependent maternal effects across the life history. *Ecology* 89, 418–427. doi: 10.1890/07-0449.1

McGhee, K. E., Pintor, L. M., Suhr, E. L., and Bell, A. M. (2012). Maternal exposure to predation risk decreases offspring antipredator behaviour and survival in threespined stickleback. *Funct. Ecol.* 26, 932–940. doi: 10.1111/j.1365-2435.2012.02008.x

R Development Core Team (2019). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Shama, L. N. S., Strobel, A., Mark, F. C., and Wegner, K. M. (2014). Transgenerational plasticity in marine sticklebacks: maternal effects mediate impacts of a warming ocean. *Funct. Ecol.* 28, 1482–1493. doi: 10.1111/1365-2435.12280

Sih, A., and McCarthy, T. M. (2002). Prey responses to pulses of risk and safety: testing the risk allocation hypothesis. *Anim. Behav.* 63, 437–443. doi: 10.1006/anbe.2001.1921

Stamps, J. A., and Krishnan, V. V. (2014). Combining information from ancestors and personal experiences to predict individual differences in developmental trajectories. *Am. Nat.* 184, 647–657. doi: 10.1086/678116

Stein, L. R., Bukhari, S. A., and Bell, A. M. (2018). Personal and transgenerational cues are nonadditive at the phenotypic and molecular level. *Nat. Ecol. Evol.* 2, 1306–1311. doi: 10.1038/s41559-018-0605-4

Tariel, J., Plénet, S., and Luquet, É (2020). Transgenerational plasticity of inducible defences: combined effects of grand-parental, parental and current environments. *Ecol. Evol.* 10, 2367–2376. doi: 10.1002/ece3.6046

Tsitrone, A., Jarne, P., and David, P. (2003). Delayed selfing and resource reallocations in relation to mate availability in the freshwater snail *Physa acuta*. *Am. Nat.* 162, 474–488. doi: 10.1086/376889

Turner, A. M., Fetterolf, S. A., and Bernot, R. J. (1999). Predator identity and consumer behavior: differential effects of fish and crayfish on the habitat use of a freshwater snail. *Oecologia* 118, 242–247. doi: 10.1007/s004420050724

Valtonen, T. M., Kangassalo, K., Pölkki, M., and Rantala, M. J. (2012). Transgenerational effects of parental larval diet on offspring development time, adult body size and pathogen resistance in *Drosophila melanogaster*. *PLoS One* 7:e31611. doi: 10.1371/journal.pone.0031611

Yin, J., Zhou, M., Lin, Z., Li, Q. Q., and Zhang, Y. (2019). Transgenerational effects benefit offspring across diverse environments: a meta-analysis in plants and animals. *Ecol. Lett.* 22, 1976–1986. doi: 10.1111/ele.13373

Zizzari, Z. V., van Straalen, N. M., and Ellers, J. (2016). Transgenerational effects of nutrition are different for sons and daughters. *J. Evol. Biol.* 29, 1317–1327. doi: 10.1111/jeb.12872

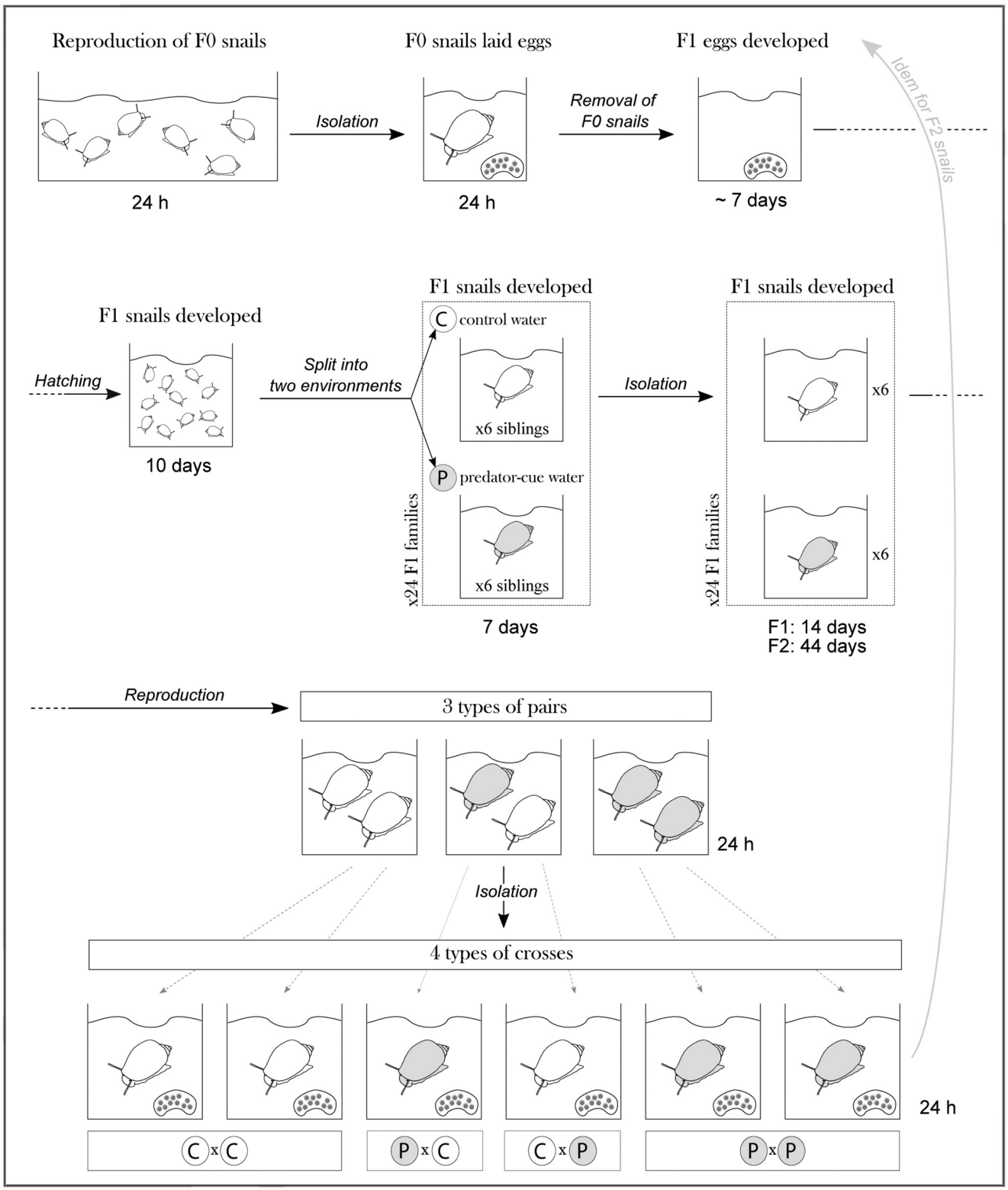
Zuccolo, L., DeRoo, L. A., Wills, A. K., Davey Smith, G., Suren, P., Roth, C., et al. (2016). Pre-conception and prenatal alcohol exposure from mothers and fathers drinking and head circumference: results from the Norwegian Mother-Child Study (MoBa). *Sci. Rep.* 6:39535. doi: 10.1038/srep39535

Zuur, A., Ieno, E. N., Walker, N., Saveliev, A. A., and Smith, G. M. (2009). *Mixed Effects Models and Extensions in Ecology with R*. New York, NY: Springer-Verlag. doi: 10.1007/978-0-387-87458-6

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDIX

Appendix 1: Experimental Design

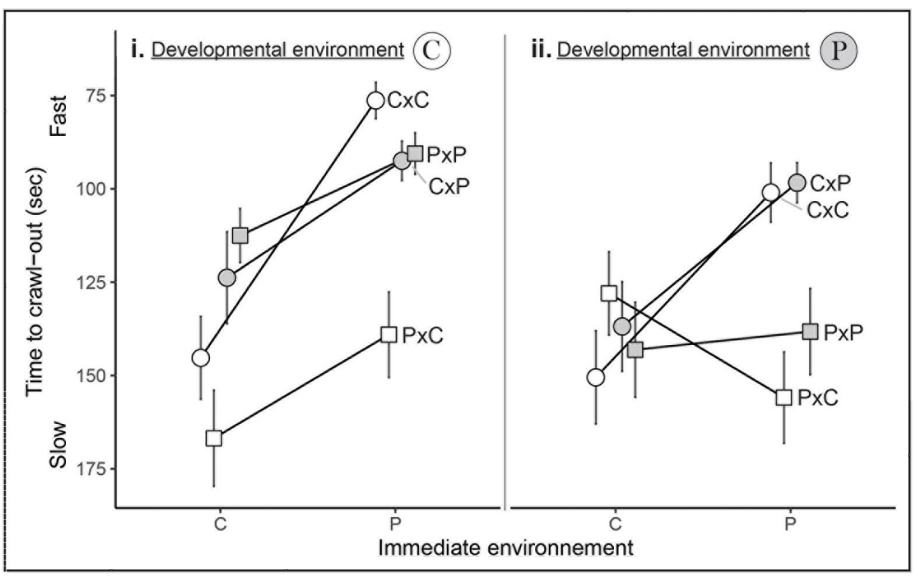


Appendix 2: Post hoc Comparisons

Note: "C" stands for control environment and "P" for predator-cue environment. "Dev" stands for developmental environment. *P*-values are corrected for multiple testing with Holm method. Bold *p*-values indicate significant pairwise comparisons (corrected *P*-value ≤ 0.05).

Trait	Immediate	Dev	contrast	Effect of:	*t*	*P*
Escape behavior	C	C	PxC-CxC	Single maternal exposure	−0.81	0.420
			CxP-CxC	Single paternal exposure	1.29	0.397
			PxP-CxC	Combined parental exposure	1.83	0.204
		P	PxC-CxC	Single maternal exposure	1.35	0.357
			CxP–CxC	Single paternal exposure	1.92	0.170
			PxP-CxC	Combined parental exposure	1.00	0.357
	P	C	PxC-CxC	Single maternal exposure	−5.33	**<0.001**
			CxP-CxC	Single paternal exposure	−1.18	0.293
			PxP-CxC	Combined parental exposure	−1.46	0.293
		P	PxC-CxC	Single maternal exposure	−3.15	**0.006**
			CxP-CxC	Single paternal exposure	−0.53	0.596
			PxP-CxC	Combined parental exposure	−2.25	**0.050**
Trait	**Immediate**	**Dev**	**contrast**	**Effect of:**	**t**	**P**
Activity	C	C	PxC-CxC	Single maternal exposure	1.31	0.383
			CxP-CxC	Single paternal exposure	2.12	0.104
			PxP-CxC	Combined parental exposure	1.07	0.383
		P	PxC-CxC	Single maternal exposure	−0.84	0.808
			CxP-CxC	Single paternal exposure	−0.41	0.808
			PxP-CxC	Combined parental exposure	2.10	0.111

Appendix 3: Effect of the Immediate Environment of Escape Behavior



Most snails escape faster in the immediate predator-cue environment ("P") than in the immediate control environment ("C"). The "C" developmental environment is shown on the left panel (i) and the "P" developmental environment is shown on the right panel (ii). Text indicates the four combinations of maternal x paternal environments (CxC, PxC, CxP, and PxP). Dots are for snails from non-exposed mothers while squares are for snails from exposed mothers. White shapes are for snails from non-exposed fathers while gray shapes are for snails from exposed fathers. Points are mean ± SE.

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Ontogenetic Basis of Among-Generation Differences in Size-Related Traits in a Polyphenic Butterfly

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Seasonal polyphenisms are cases in which individuals representing generations occurring in different times of the year systematically differ in their morphological, physiological, and/or behavioral traits. Such differences are often assumed to constitute adaptive responses to seasonally varying environments, but the evidence for this is still scarce. The adaptive character of the response would be corroborated by the pattern in which the decision about choosing a particular seasonal phenotype is made before the onset of respective environmental conditions (anticipatory plasticity). Alternatively, the between-generation differences can be caused by immediate effects of seasonally varying environments (responsive plasticity). Here we reared the larvae of the seasonally polymorphic map butterfly *Araschnia levana* under two different photoperiodic regimes, which provided different seasonal cues. These two treatments induced direct development and diapause pathways, respectively. Replicating the experiment at different temperatures and levels of host plant quality allowed us to evaluate both the anticipatory and the responsive components of the associated plastic changes in life-history traits. Larvae representing the direct development pathway invariably had higher growth rates and shorter development periods, although the difference between the developmental pathways was smaller at inferior host quality. Body size differences between the developmental pathways turned out to be less consistent, as the natural pattern of higher pupal mass of the directly developing individuals could only be reproduced at lower rearing temperature. Though being considerably modified by immediate environmental effects, the between-generation differences in size, growth rates, and larval are largely based on anticipatory plasticity (= responses to photoperiodic cues) and should be treated as seasonal adaptations in *A. levana*. In a more general context, we show how investigating the proximate basis of size differences can serve the purpose of identifying the limits of phenotypic plasticity in juvenile growth schedules.

Keywords: reaction norms, age and size at maturity, photoperiodism, overwintering, Nymphalidae, insects, sexual size dimorphism

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INTRODUCTION

Reaction norms for size and time at maturation constitute a core element of the theory of life-history evolution (Stearns, 1992; Roff, 1992, 2002). Evolutionary forces shaping such reaction norms can perhaps be best understood through comparisons among groups such as closely related species (Tammaru et al., 2015; Kivelä et al., 2020), different populations within species (Meister et al., 2017a,b, 2018), and sexes (Teder and Tammaru, 2005). Additionally, the numerous well-known examples of insect polyphenism in general (reviewed by Shapiro, 1976; Simpson et al., 2011; Nylin, 2013) and seasonally polyphenic insects in particular offer an excellent opportunity to compare distinct morphs, which often differ in values of developmental traits (Wiklund et al., 1991; Teder et al., 2010; Morehouse et al., 2013; Kivelä et al., 2015). An obvious advantage of such studies is that the groups to be compared can be considered genetically identical, so that any observed differences can unambiguously be ascribed to phenotypic plasticity.

A particular question to be answered in studies on such reaction norms is that about the proximate basis of the differences in body sizes. The individuals that ultimately attain larger adult sizes may (1) be larger from the beginning, (2) grow for a longer time, or (3) grow faster (Blanckenhorn, 2000; Sõber et al., 2019). As one particular implication, the answer to this question has direct relevance to the problem of evolutionary ecology of growth rate as a separate life-history trait (Arendt, 1997). If the differences in final sizes are due to growth rates, revealing the associated differences in other traits allows us to address the question about costs of fast growth (Dmitriew, 2011). In turn, if the groups being compared do not differ in growth rate, this may indicate that growth is indeed maximized within limits set by physiology, as the classic life-history models often assume (Stearns, 1992; Roff, 1992).

Another proximate level question is how early—in the ontogeny of the individual—the alternative developmental pathways diverge from each other. The answer to this question can contribute to identifying the limits to adaptive evolution of juvenile growth patterns. In particular, in the cases of sexual size dimorphism, the sexes start to diverge already in early phases of larval development (see Stillwell et al., 2014; Vendl et al., 2018; Chelini et al., 2019 for recent studies). This pattern has been interpreted as evidence of some constraint on major sex-related changes late in larval development: differences in adult size cannot be attained in one step but have to be accumulated throughout the development (Tammaru et al., 2010; Meister et al., 2018).

From a different—ultimate rather than proximate—perspective, knowing when and how do size differences appear during immature development can shed light on adaptive nature of the divergent growth patterns (Esperk et al., 2013). Different seasonal generations of insects pass through their immature development in predictably differing environmental conditions, with temperature and food (=host plant, for herbivorous insects) quality being perhaps the most significant seasonally variable parameters. Both temperature and host quality have strong direct effects on larval growth schedules, which may not require any explanation beyond the proximate physiological one (responsive plasticity sensu Whitman and Agrawal, 2009). On the other hand, in seasonal environments, the stages succeeding the larval stage can experience predictably different conditions as well. In such cases, the growing immatures are expected to react to cues of forthcoming environmental changes in a way shaped by natural selection. For example, short photoperiod is frequently used as a cue of the onset of the unfavorable season, which primarily informs developmental decisions related to winter diapause (Tauber et al., 1986; Danks, 1987; Leather et al., 1993; Koštál, 2006; Saunders, 2020). This type of response to environmental factors has been termed *anticipatory plasticity* and should be considered adaptive (Whitman and Agrawal, 2009).

In the present study, we make use of a comparison of growth patterns between two seasonal generations of a polyphenic butterfly. The two different developmental pathways—direct and diapause development—were induced in the laboratory by manipulating photoperiodic regime. The experiment was replicated under two temperatures and in different times of the year (=different host quality). The larvae were weighed daily to obtain detailed information about their growth curves in different treatments. This allowed us to treat development periods, final masses of instars, and growth rates of individual larvae as separate response variables and to assess plastic responses in all these parameters. The detailed information accumulated facilitated evaluating different adaptive hypotheses, as well as shed light on proximate basis of plastic responses in insect growth schedules.

From the ultimate perspective, the used three-factor design allowed us to explicitly compare effects attributable to responsive vs. anticipatory plasticity. In particular, we proceeded from the assumption that any responses to the photoperiodic cue (especially when consistent across other treatments) would likely have the anticipatory character (Esperk et al., 2013) and used the opportunity to compare the magnitude of such (presumably adaptive) effects to the physiologically based responses to temperature and food quality. The latter are rather universal in insects and should be represented by lower growth rates, longer development times, and lower final body masses as a response to inferior food quality (Teder et al., 2014) and lower growth rates, longer development times, but higher final masses under lower temperatures (Atkinson, 1994, 1996; Atkinson and Sibly, 1997; Angilletta et al., 2004).

MATERIALS AND METHODS

Study Species

The European map butterfly (*Araschnia levana* L., Lepidoptera: Nymphalidae) is a temperate Palaearctic insect species that is bivoltine in most of its range (including the study area), although a facultative third generation may occur in the southern parts of its distribution area (Tolman and Lewington, 2008). The species is well known for its striking seasonal polyphenism in wing color and patterning (Friberg and Karlsson, 2010; Ihalainen and Lindstedt, 2012). The between-generation difference in life span (mainly attributable to the different duration of

the pupal stage, which is the overwintering stage in this species) is no less impressive, as completing of the life cycle takes approximately 10 weeks in directly developing generation compared to 42 weeks of the diapausing one (Freitak et al., 2019). In addition, the two generations are known to differ in adult abundance (Viidalepp and Remm, 1996), body composition and resource allocation patterns (Friberg and Karlsson, 2010; Morehouse et al., 2013), body design and flight performance (Fric and Konvička, 2002; Friberg and Karlsson, 2010), and immunity (Baudach et al., 2018; Freitak et al., 2019) and body size (about 15% in terms of live mass of the pupae, Freitak et al., 2019; personal observations of the authors). The eggs are laid in string-like clusters, and the larvae aggregate on their host plant (*Urtica* spp.), whereas the group size declines with larval ontogeny (Ruf, 2002). Like in most temperate insects, seasonal polyphenism in *A. levana* is controlled primarily by photoperiod: long days during larval stage induce the directly developing form, whereas short-day conditions lead to the diapausing pathway. The decision whether to develop directly or overwinter in the pupal stage is made during the 4th and 5th (i.e., penultimate and final, respectively) larval instars (Friberg et al., 2011).

Experimental Design

To investigate the influence of environmental factors on the life-history traits of the two developmental pathways, map butterfly larvae were reared in controlled conditions under a 2 × 2 × 2 crossed experimental design (**Supplementary Table 1**). The factors varied were (1) photoperiod—long (18L:6D, inducing direct development) or short day (12L:12D, inducing diapause development), (2) temperature (17 vs. 22°C), and (3) host quality, manipulated by means of rearing the larvae in either early (June) or late season (August).

The progeny of map butterfly females of Estonian origin was transferred to 50-mL plastic vials in groups of 10 newly hatched larvae. The vials were then equally divided between the two rearing chambers set to different photoperiodic regimes, considering equal representation of broods (split-family design) and hatching dates in the treatments. To induce different developmental pathways, one of the chambers was set to the long-day conditions (18L:6D), whereas in the other the short-day conditions (12L:12D) were created. To reduce the potential microclimatic differences of the rearing chambers on larval growth, the photoperiodic treatments (and respective larvae) were rotated between the chambers with a 3-day interval. Leaves of the stinging nettle (*Urtica dioica*) were provided as food and were changed every other day or more frequently when necessary to avoid food depletion.

The larvae were reared in groups until the end of their 3rd instar being housed individually in the vials of the same type later on. In the cases when 6 or more larvae out of 10 died in the vial before the end of their 3rd instar, the survivors were divided between the vials representing the same brood and the same hatching date. From the beginning of the 4th instar onward, the larvae were weighed daily until pupation.

The experiments were performed in 2 years (2010 and 2011) at the University of Tartu, Estonia. To investigate the influence of food quality on larval development, the larvae were reared both in early season (June, the time when directly developing generation is in their larval stage) and in late season (August, the time for the larval stage of diapausing generation). The progeny of wild-collected diapausing individuals was used in all early season experiments, while the offspring of wild-collected directly developing individuals was used in late season experiments. As the quality of nettle leaves has been shown to decline with progressing season (Morehouse et al., 2013), the larvae from the early season rearings were assumed to receive high-quality diet, whereas the diet quality was lower in late season. In 2010, the larvae were reared at 22°C, while the temperature was set to 17°C in 2011.

To examine the proximate effect of light conditions on larval growth rates, the larvae were weighed twice a day during the first 2 days in both early and late season experiment in 2011. Specifically, 12 hours' weighing interval was targeted (but it varied from 10 to 14 h, for technical reasons). As a result, the "diurnal period" covered only photophase of both photoperiodic treatments, whereas the "nocturnal period" included only scotophase of the short-day treatment and 6 h photophase, followed by 6-h scotophase of the long-day treatment.

Data Analysis

The dependence of development times (durations of the 4th and 5th instar), body masses and growth rates on developmental pathway (direct/diapause), temperature (17/22°C), season (early season/late season), sex (male/female), and their interaction were analyzed by general linear mixed models (PROC MIXED, Littell et al., 2006; data of different experiments pooled). Brood was included as a random factor; denominator degrees of freedom were estimated using the Satterthwaite option. Minimum adequate models were constructed by sequentially removing non-significant interaction terms.

Instar durations were expressed as the time between the two successive molts. Alternatively, active growth phases were calculated based on the time of preceding molt and the time of attaining the peak mass of an instar. Growth rates were calculated as (mass at the end of the period$^{1/3}$ − mass at the beginning of the period$^{1/3}$)/(duration of the period) (Esperk and Tammaru, 2004; Tammaru and Esperk, 2007). Two different indices of growth rate were derived. First, *instantaneous growth rates* were calculated for a 24-h period on the 2nd day of the instar (the "free growth period," Esperk and Tammaru, 2004; Meister et al., 2017a). Second, *integral growth rates* were found for the entire positive growth phase of the instar (from the molt until the attainment of peak body mass). In experiments in which the effect of light conditions on larval growth was studied, the observation periods deviating from the targeted 12-h period were corrected according to the actual time span. The larvae that died before the pupation (3–9% mortality during the last two instars in different trials, with no substantial differences between the treatments) and the small proportion of insects (5%, on average) that entered the pupal diapause in the long-day photoperiodic treatments were discarded from further analyses. All analyses were conducted in SAS 9.4 (SAS Institute Inc., Cary, NC, United States).

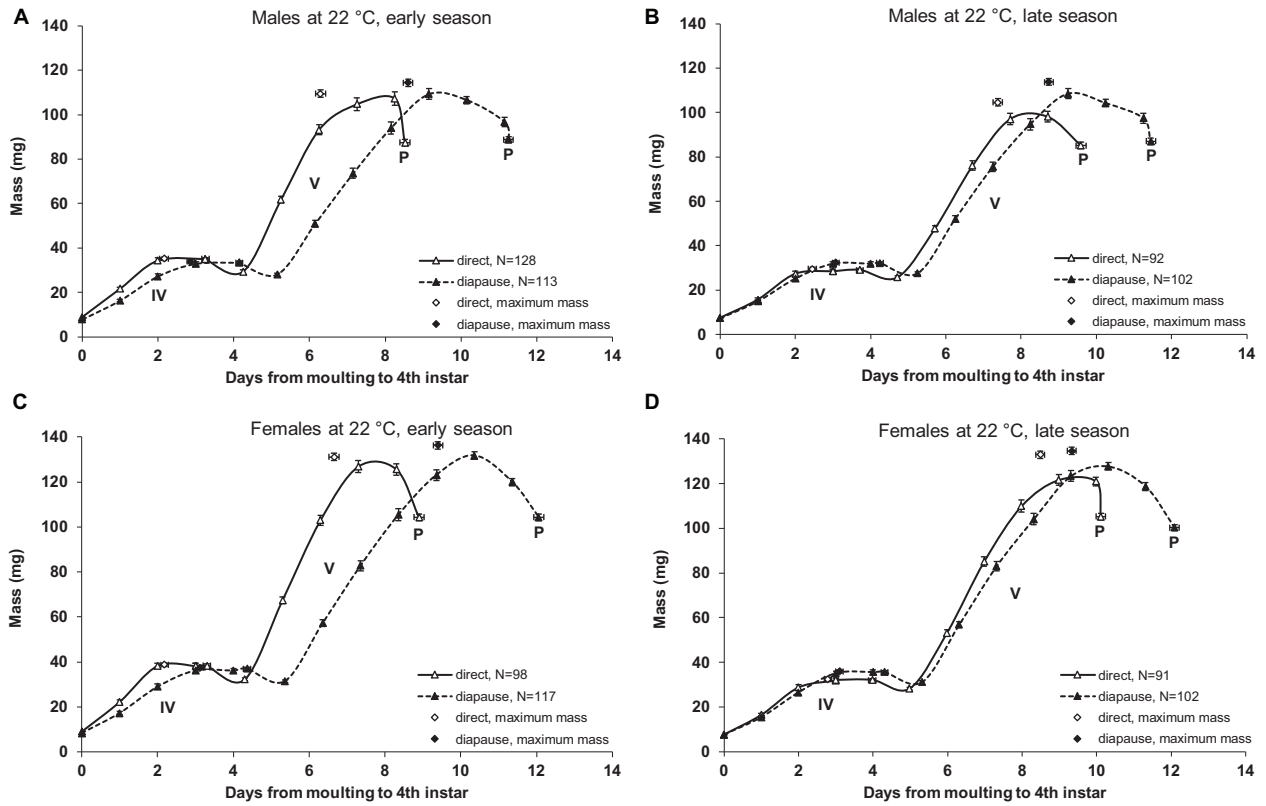

FIGURE 1 | Growth curves of *Araschnia levana* larvae reared at 22°C in 2010. **(A)** Male larvae reared in early season (June). **(B)** Male larvae reared in late season (August). **(C)** Female larvae reared in early season. **(D)** Female larvae reared in late season. Direct development ["long day" (18L:6D) photoperiodic treatment] and diapause ["short day" (12L:12D) photoperiodic treatment] pathways were induced. Values are corrected for the effect of brood (using SAS, PROC MIXED, least square means option, Littell et al., 2006). Roman numerals stand for instars, "P" indicates pupation, and "N" marks sample sizes in the beginning of the instars and in the pupal stage. Error bars indicate standard errors; horizontal error bars stand for standard errors for the duration of the 4th and 5th instar and for the time period from molting until attaining peak body mass in respective instar.

RESULTS

There was a consistent effect of photoperiod on development time (**Figures 1**, **2** and **Supplementary Table 2**). In particular, the time spent in both penultimate and final instar was 10–20% longer in individuals that developed through diapause pathway, compared to directly developing individuals. The magnitude of the difference varied, depending on temperature and season but invariably attained statistical significance (**Table 1** and **Figures 1**, **2**). The alternative measure of development time, the period of active growth (from molting until achieving of peak body mass), was also longer in diapausing than in directly developing individuals (**Table 2**). Individuals reared at 17°C had, on average, 50% longer penultimate and final instar durations and longer active growth phases than those reared at the higher temperature; late-season larvae had 5–10% longer instar durations than those reared in early season (**Supplementary Table 2**), and instar durations of females were 5–15% longer than in males (all differences statistically significant, **Tables 1**, **2**). The differences in durations of the 4th and 5th instar between the developmental pathways were larger in early than in late season and at lower than at higher rearing temperatures (significant interaction terms, **Tables 1**, **2** and **Supplementary Table 2**). However, when analyzed separately by rearing temperature and season, the differences between developmental pathways remained significant in all cases except in the 4th instar active growth phase of larvae reared at low temperature and in late season. In the final instar, the difference between the sexes was higher at lower than at higher rearing temperature and in late than in early season (significant interaction terms, **Tables 1**, **2**).

There was a consistent effect of photoperiod on larval growth rates (**Figures 1**, **2** and **Supplementary Table 2**). Instantaneous and integral growth rates, both in the 4th and 5th instar, were higher in directly developing than in diapausing individuals and in individuals reared at the higher than at the lower temperature (**Tables 3**, **4** and **Supplementary Table 2**). In both instars, integral growth rates were higher in early than in late season (**Table 4**); however, there were no seasonal differences in instantaneous growth rates (**Table 3**). There was no significant sexual difference in growth rates in the 4th instar, but in the 5th instar, females had higher instantaneous growth rates and lower integral growth rates than males (**Tables 3**, **4**). There was a larger difference in integral growth rates between developmental pathways at higher than at lower temperature and in early than in late season

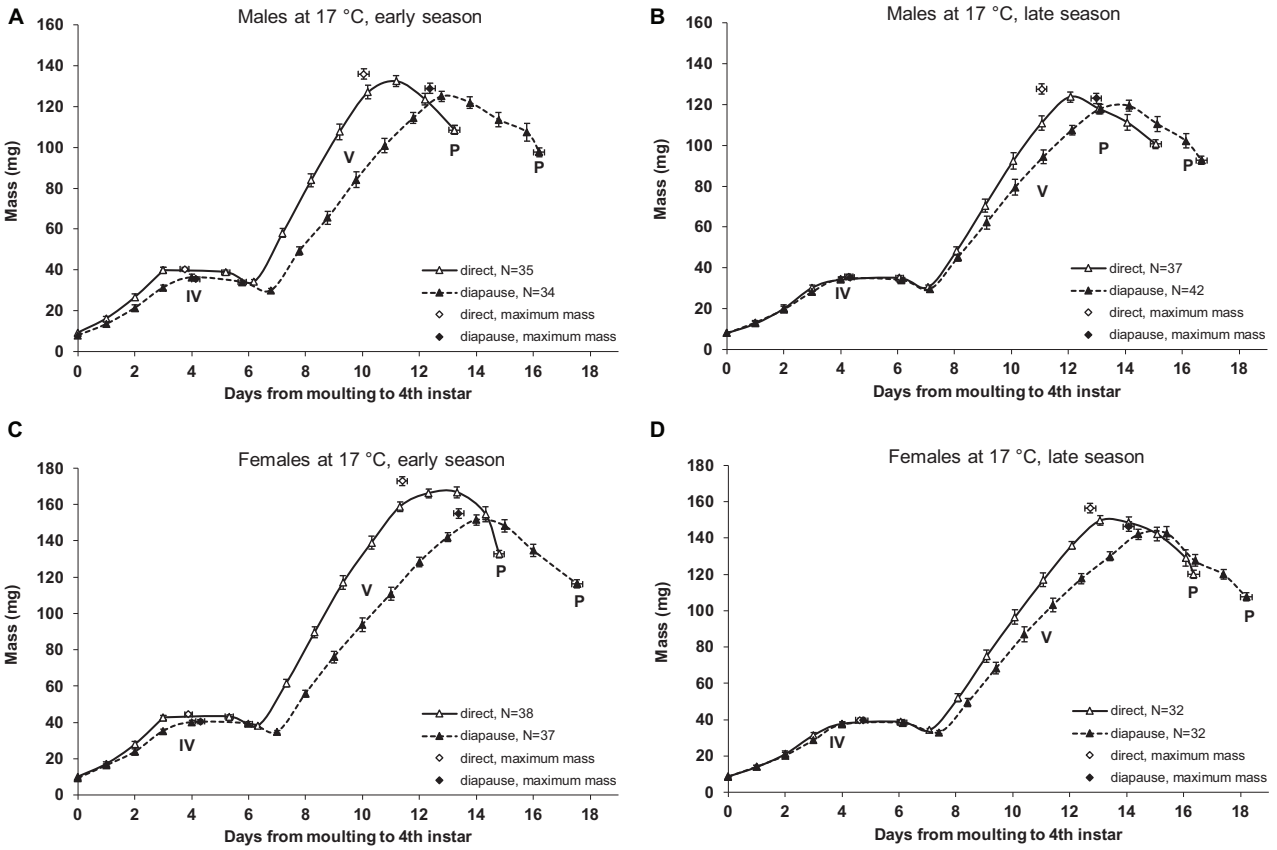

FIGURE 2 | Growth curves of *Araschnia levana* larvae reared at 17°C. Direct development ["long day" (18L:6D) photoperiodic treatment] and diapause ["short day" (12L:12D) photoperiodic treatment] pathways were induced in 2011. **(A)** Male larvae reared in early season. **(B)** Male larvae reared in late season. **(C)** Female larvae reared in early season. **(D)** Female larvae reared in late season. Direct development and diapause pathways were induced. See **Figure 1** for details.

(significant interaction terms, **Table 4**). When analyzed separately by rearing temperature and season, the differences in growth rates between the developmental pathways remained significant in all cases with one exception (4th instar integral growth rate at low temperature in late season).

Photoperiod had an influence on body sizes, but interactive effects were substantial (**Figures 1**, **2** and **Supplementary Table 2**). In 2011 experiments in which the proximate effect of light conditions on growth was studied, directly developing individuals had higher instantaneous growth rates during the 4th instar 2nd-day diurnal period (30% higher growth rates; $F_{1,257} = 46.0$, $P < 0.0001$) and the 5th instar 2nd-day diurnal period (30% higher growth rates; $F_{1,269} = 51.9$, $P < 0.0001$) than diapause-destined individuals. However, the growth rates did not differ significantly between the developmental pathways during the 2nd night (i.e., nocturnal phase of the second day) in either 4th instar ($F_{1,265} = 2.2$, $P = 0.14$) or 5th instar ($F_{1,261} = 3.1$, $P = 0.077$).

Pupal masses were 5% higher in diapausing than in directly developing individuals, 15% higher at lower than at higher rearing temperature, 5% higher in early than in late season (**Supplementary Table 2**), and 20% higher in females than in males (all differences statistically significant, **Table 5** and **Figures 1**, **2**). However, when analyzed separately by rearing temperature (justified by significant interaction term, **Table 5**), directly developing individuals achieved significantly (10%) higher pupal masses only at 17°C, whereas there were no significant differences in pupal mass between developmental pathways at 22°C (**Supplementary Table 2**).

Premolt masses and peak masses in the 4th and 5th instar were significantly higher in in early than in late season and, with the exception of the 4th instar premolt mass, at lower than at higher rearing temperatures (**Tables 6**, **7**, **Supplementary Table 2**, and **Figures 1**, **2**). However, the difference in both penultimate and last instar masses between the developmental pathways was strongly influenced by rearing conditions (indicated by significant interaction terms between developmental pathway and rearing temperature and between developmental pathway and season, **Tables 6**, **7** and **Supplementary Table 2**). In particular, in the 4th instar and in the beginning of the 5th instar, directly developing individuals were heavier than diapausing individuals only in the early season, whereas diapausing individuals achieved higher masses in late season (**Tables 6**, **7** and **Figures 1**, **2**). Consistently, body mass in the 5th instar and peak mass in the 4th instar was higher in directly developing than in diapausing individuals only at 17°C, whereas

diapausing individuals were heavier than directly developing ones at 22°C.

DISCUSSION

Our results demonstrate that the two seasonal generations of the European map butterfly differ in various parameters of larval growth schedules. The seasonal generations (directly developing vs. diapause-destined individuals) were induced experimentally by photoperiodic treatments. Most clearly and consistently, there were considerable among-generation differences in instar-specific development times—the larvae that were to produce diapausing pupae spent more time in both final and penultimate larval instars. At low (but not high) temperatures, the diapause development larvae also attained lower pupal masses. Directly developing larvae had higher growth rates than those heading toward diapause, consistently so across different rearing conditions, sexes, and larval instars, irrespectively of the way how the growth rates were expressed (instantaneous or integral). However, in more detailed comparisons, instantaneous growth rates of the developmental pathways significantly differed only during the diurnal but not in the nocturnal phase.

Our results show that the between-generation differences in developmental schedules contain a considerable element of anticipatory plasticity and can therefore be considered adaptive.

TABLE 1 | Duration of the 4th and 5th instar of *Araschnia levana* larvae as explained by developmental pathway (direct vs. diapause development), temperature (17 vs. 20°C), season (June vs. August), and sex; linear mixed models (SAS, Proc MIXED, type 3 sums of squares) with brood (progeny of one female) as a random effect.

	Duration of 4th instar			Duration of 5th instar		
Effect	***df***	***F***	***P***	***df***	***F***	***P***
(D)evelopmental pathway[a]	1, 1034	221.2	<0.0001	1, 1080	1046.0	<0.0001
(T)emperature[b]	1, 29.2	377.6	<0.0001	1, 28.7	638.7	<0.0001
(S)eason[c]	1, 28.3	15.9	0.0004	1, 27.5	15.1	0.0006
Sex[d]	1, 1020	23.5	<0.0001	1, 1067	368.8	<0.0001
D × T[e]	1,1034	7.0	0.0083	1, 1080	19.2	<0.0001
D × S[f]	1, 1026	44.9	<0.0001	1, 1071	62.9	<0.0001
T × sex[g]				1, 1067	49.6	<0.0001
S × sex[h]				1, 1066	9.6	0.002
D × S × sex[i]				2, 1067	6.7	0.013

The two developmental pathways were induced by different photoperiodic treatments, corresponding to the two seasonal generations. Only individuals that survived until pupation were included into the analyses. Degrees of freedom were estimated by the Satterthwaite method. Full models were simplified so that only interactions statistically significant at the 0.05 level are included.

[a]Longer 4th and 5th instar in diapausing than in directly developing individuals.
[b]Longer 4th and 5th instar at lower temperature than at higher temperature.
[c]Longer 4th and 5th instar in late than in early season.
[d]Longer 4th and 5th instar in females than in males.
[e]The difference in the duration of the 4th and 5th instar between developmental pathways was larger at higher than at lower temperature.
[f]The difference in duration of the 4th and 5th instar between developmental pathways was larger in early than in late season.
[g]The difference in the duration of 5th instar between the sexes was larger at lower than at higher temperatures.
[h]The difference in duration of the 5th instar between the sexes was larger in late than in early season.
[i]The sexual difference in the duration of 5th instar between developmental pathways was larger in late than in early season.

TABLE 2 | Duration of active growth phase (from molt until attainment of peak body mass) in the 4th and 5th instar *Araschnia levana* larvae as explained by developmental pathway, temperature, season, and sex; linear mixed models (SAS, Proc MIXED, type 3 sums of squares) with brood (progeny of one female) as a random effect.

	4th instar			5th instar		
Effect	***df***	***F***	***P***	***df***	***F***	***P***
(D)evelopmental pathway[a]	1, 1018	91.1	<0.0001	1, 1063	578.7	<0.0001
(T)emperature[b]	1, 29.9	233.4	<0.0001	1, 27.3	304.3	<0.0001
(S)eason[c]	1, 28.7	13.0	0.0012	1, 27.3	11.2	0.0024
Sex[d]	1, 1003	21.6	<0.0001	1, 1049	253.5	<0.0001
D × T[e]	1,1018	18.3	<0.0001	1, 1063	8.6	0.0035
D × S[f]	1, 1011	18.4	<0.0001	1, 1063	26.3	<0.0001
T × sex[g]				1, 1049	23.4	<0.0001
S × sex[h]				1, 1049	4.6	0.032
D × T × S[i]				2, 51.4	5.0	0.011
D × S × sex[j]				2, 1050	4.4	0.013

*See **Table 1** for further details.*
[a]Longer in diapausing than in directly developing individuals.
[b]Longer at lower than at higher temperature.
[c]Longer in late than in early season.
[d]Longer in females than in males.
[e]The difference between developmental pathways was larger at higher than at lower temperature.
[f]The difference between developmental pathways was larger in early than in late season.
[g]The difference between sexes was larger at lower than at higher temperatures.
[h]The difference between sexes was larger in late than in early season.
[i]The seasonal difference between developmental pathways was larger at higher than at lower temperature.
[j]The sexual difference between developmental pathways was larger in late than in early season.

TABLE 3 | Instantaneous growth rates (in second day of the instar) in the 4th and 5th instar *Araschnia levana* larvae as explained by developmental pathway, temperature, season, and sex; linear mixed models (SAS, Proc MIXED, type 3 sums of squares) with brood (progeny of one female) as a random effect.

	4th instar			5th instar		
Effect	***df***	***F***	***P***	***df***	***F***	***P***
(D)evelopmental pathway[a]	1, 1011	36.1	<0.0001	1, 1060	218.4	<0.0001
(T)emperature[b]	1, 30.6	61.0	<0.0001	1, 27.8	54.0	<0.0001
(S)eason	1, 30.7	3.8	0.061	1, 25.8	0.5	0.49
Sex[c]	1, 1005	0.0	0.88	1, 1052	9.1	0.0026
T × S[d]	1, 30.7	4.9	0.034			
T × sex[e]	1, 1005	12.1	0.0005			

*See **Table 1** for further details.*
[a]Higher in directly developing individuals.
[b]Higher at higher than at lower temperature.
[c]Higher in the 5th instar females than in males.
[d]No seasonal difference at higher temperature, but early season larvae had higher growth rates than late season larvae at lower temperature.
[e]Females had higher growth rates than males at higher temperature, but males had higher growth rates than females at lower temperature.

This conclusion is based on the observation that some of such differences, induced here by photoperiodic treatments, consistently appeared in all combinations of the two other environmental factors manipulated in the experiment. The between-generation differences cannot thus solely be based on proximate effects of temperature and host quality during larval development. Nevertheless, both temperature and host quality still had an influence on parameters of larval growth schedules, as well as showed numerous interactive effects with the developmental pathway. In part, the lower body size of the spring-flying generation of the map butterfly can thus still be based on responsive (non-adaptive) effects of inferior food quality the larvae encounter at the end of summer, with some additional impact of temperatures experienced during larval development. Overall, in our experiments, the effects of host quality (season) tended to be somewhat weaker (for development time and growth rates) or similar (for body mass) to those of developmental pathway (**Figures 1**, **2** and **Supplementary Table 2**). In contrast, the effect of temperature was larger than the difference between developmental pathways. Nevertheless, we have to consider that the difference between the temperature treatments (5°C) considerably exceeded the differences expected between natural developmental periods of the two seasonal generations.

Of the interactive effects, we find it notable that the differences between developmental pathways were clearly more pronounced on high-quality spring food compared to the lower-quality nettle leaves offered in August. We see here an interesting parallel to the observation that, in sexually size dimorphic species, also the sexual size dimorphism (SSD) is more pronounced in favorable environmental conditions (Teder and Tammaru, 2005; Stillwell and Davidowitz, 2010).

Photoperiod is the factor that is most commonly used as a cue for forthcoming seasons by temperate insects (Beck, 1980; Saunders, 2020). Responses to photoperiod are therefore

TABLE 4 | Integral growth rates (calculated as peak mass of instar$^{1/3}$ − mass at the beginning of the instar$^{1/3}$)/(duration of the period from molt to attainment of peak body mass] in the 4th and 5th instar *Araschnia levana* larvae as explained by developmental pathway, temperature, season, and sex; linear mixed models (SAS, Proc MIXED, type 3 sums of squares) with brood (progeny of one female) as a random effect.

	4th instar			5th instar		
Effect	***df***	***F***	***P***	***df***	***F***	***P***
(D)evelopmental pathway[a]	1, 1014	81.9	<0.0001	1, 1059	416.4	<0.0001
(T)emperature[b]	1, 30.7	106.6	<0.0001	1, 27.2	165.6	<0.0001
(S)eason[c]	1, 29.6	18.7	0.0002	1, 27.2	10.0	0.0038
Sex[d]	1, 1000	0.5	0.46	1, 1044	10.4	0.0013
D × T[e]	1,1014	26.0	<0.0001	1, 1059	22.1	<0.0001
D × S[f]	1, 1007	47.7	<0.0001	1, 1059	25.8	<0.0001
D × T × S[g]				2, 51.2	7.0	0.002

*See **Table 1** for further details.*
[a]Higher in directly developing than in diapausing individuals.
[b]Higher at higher than at lower temperature.
[c]Higher in early than in late season.
[d]Higher in the 5th instar males than females.
[e]Higher difference between developmental pathways at higher than at lower temperature.
[f]Higher difference between developmental pathways in early than in late season.
[g]Seasonal difference between developmental pathways in the 5th instar growth rates was higher at higher than at lower temperature.

TABLE 5 | Pupal mass of *Araschnia levana* as explained by developmental pathway, temperature, season, and sex; linear mixed models (SAS, Proc MIXED, type 3 sums of squares) with brood (progeny of one female) as a random effect.

Effect	***df***	***F***	***P***
(D)evelopmental pathway[a]	1, 1080	104.5	<0.0001
(T)emperature[b]	1, 26.4	87.3	<0.0001
(S)eason[c]	1, 26.4	11.3	0.0024
Sex[d]	1, 1066	920.7	<0.0001
D × T[e]	1, 1080	91.8	<0.0001
D × sex[f]	1, 1066	16.5	<0.0001
T × S[g]	1, 26.4	4.6	0.041
T × sex[h]	1, 1066	6.0	0.015
D × T × S[i]	2, 1073	3.2	0.042

*See **Table 1** for further details.*
[a]Higher in directly developing than diapausing individuals.
[b]Higher at lower than at higher temperature.
[c]Higher in early than in late season.
[d]Higher in females than in males.
[e]No difference between the developmental pathways at higher temperature, at lower temperature directly developing individuals formed heavier pupae than diapausing individuals.
[f]The sexual difference was larger in directly developing than in diapausing individuals.
[g]The seasonal difference was larger at lower than at higher temperatures.
[h]The sexual difference was larger at lower than at higher temperature.
[i]The seasonal difference between developmental pathways was larger at lower than at higher temperature.

TABLE 6 | Fourth instar premolt mass (mass before the molt to the 4th instar) and 4th instar peak mass of *Araschnia levana* larvae as explained by developmental pathway, temperature, season, and sex; linear mixed models (SAS, Proc MIXED, type 3 sums of squares) with brood (progeny of one female) as a random effect. See **Table 1** for further details.

	4th instar premolt mass			4th instar maximal mass		
Effect	***df***	***F***	***P***	***df***	***F***	***P***
(D)evelopmental pathway[a]	1, 1018	22.1	<0.0001	1, 1014	7.6	0.006
(T)emperature[b]	1, 28.2	1.1	0.31	1, 28.7	31.7	<0.0001
(S)eason[c]	1, 27.7	22.8	<0.0001	1, 28.1	20.0	0.0001
Sex[d]	1, 1015	41.8	<0.0001	1, 1003	244.9	<0.0001
D × T[e]				1, 1014	36.4	<0.0001
D × S[f]	1, 1014	34.1	<0.0001	1, 1007	79.3	<0.0001

[a]Higher in directly developing than diapausing individuals.
[b]Higher at lower than at higher temperature.
[c]Higher in early than in late season.
[d]Higher in females than in males.
[e]Peak mass of directly developing individuals was higher than in diapausing individuals at lower temperature, but diapausing individuals had higher peak body mass than directly developing ones at higher temperature.
[f]Mass of directly developing individuals was higher than in diapausing individuals in early season, but diapausing individuals had higher mass than directly developing ones in late season.

TABLE 7 | Fifth instar premolt mass (mass before the molt to the 5th instar) and 5th instar peak mass of *Araschnia levana* larvae as explained by developmental pathway, temperature, season, and sex; linear mixed models (SAS, Proc MIXED, type 3 sums of squares) with brood (progeny of one female) as a random effect.

	5th instar premolt mass			5th instar maximal mass		
Effect	***df***	***F***	***P***	***df***	***F***	***P***
(D)evelopmental pathway[a]	1, 1075	11.3	0.0008	1, 1071	10.6	0.0011
(T)emperature[b]	1, 28.6	23.6	<0.0001	1, 25.7	145.4	<0.0001
(S)eason[c]	1, 27.8	20.7	<0.0001	1, 25.8	10.3	0.0035
Sex[d]	1, 1063	265.1	<0.0001	1, 1056	1248.8	<0.0001
D × T[e]	1,1075	47.0	<0.0001	1, 1071	97.2	<0.0001
D × S[f]	1, 1011	96.3	<0.0001			
D × sex[g]				1, 1056	16.5	<0.0001
T × S[h]				1, 25.8	5.3	0.03
T × sex[i]				1, 1056	13.9	0.0002

*See **Table 1** for further details.*
[a]*Higher in directly developing than diapausing individuals.*
[b]*Higher at lower than at higher temperature.*
[c]*Higher in early than in late season.*
[d]*Higher in females than in males.*
[e]*Directly developing individuals were heavier than diapausing individuals at lower temperature, but diapausing individuals had higher mass than directly developing ones at higher temperature.*
[f]*In early season directly developing individuals had higher premolt mass than diapausing ones while the opposite was true in late season.*
[g]*No difference between developmental pathways in maximal mass in the 5th instar males, whereas directly developing females achieved higher 5th instar peak body mass than diapausing females.*
[h]*The seasonal difference was larger at lower temperature.*
[i]*The sexual difference was larger at lower than at higher temperature.*

commonly considered to have an anticipatory character but they may nevertheless include elements of responsive plasticity. Indeed, in several insects, feeding behavior and growth patterns have been shown to differ between the day and the night, typically in the way that larvae feed more actively and have higher growth rates at night (Berger and Gotthard, 2008; Berger et al., 2011). Surprisingly, however, in our experiments, directly developing larvae had higher growth rates than diapause-destined larvae during the diurnal, but not during the nocturnal phase of the active growth period in both penultimate and final instar. As conditions during the diurnal phase were exactly the same for both developmental pathways, these differences can only be ascribed to anticipatory and not to responsive plasticity. Thus, the response to the photoperiod very likely has an adaptive character in *A. levana*. On the other hand, it cannot be excluded that responses of larval growth to temperature and food quality can also contain anticipatory/adaptive elements, rather than being entirely based on some universal physiological relationships. In our opinion, such hypotheses should best be approached through cross-species comparison revealing phylogenetically conserved—physiologically based—elements of reaction norms and the effects that range beyond those (Tammaru et al., 2015; Kivelä et al., 2020).

Adaptive value of the seasonal differences in developmental schedules of *A. levana* is not obvious (see Morehouse et al., 2013, for discussion). On average, in the field, the individuals of the diapausing generation are clearly smaller (Reinhardt, 1984; personal observations of the authors from the study area) than their directly developing offspring, which have their adult period in summer. As the adaptive explanation, it has been proposed that summer generation adults of *A. levana* (like several other butterflies) are selected for higher mobility (Fric and Konvička, 2002; Fric et al., 2006). Additionally, seasonal differences in larval mortality rates have a strong potential to affect generation-specific optima in body sizes: under high predation risk, attempting to grow large should be selected against because both of increased cost of spending more time in the vulnerable larval stage and the higher cost of being large (and therefore more apparent) *per se* (Remmel and Tammaru, 2009; Teder et al., 2010; Remmel et al., 2011). This explanation is, however, hardly applicable to explain seasonal differences in body size in *A. levana* as the larvae attain larger sizes when growing in June, which is the time when overall larval mortality rates are expected to peak in boreal forest landscapes (Remmel et al., 2009). The mortality hypothesis could, nevertheless, be used to explain the longer developmental periods of the diapausing individuals: it is most likely safer to spend a few extra days as a larva in late August compared to June. The benefits of having a longer development period in the diapausing individuals can be associated with acquiring immunological advantage (as shown for *A. levana* in particular: Vilcinskas and Vogel, 2016; Baudach et al., 2018; Freitak et al., 2019) or facilitation of other physiological processes needed to prepare the insect for an 8-month-long diapause (Hahn and Denlinger, 2007, 2011; Wang et al., 2007; Lehmann et al., 2016).

In the lower temperature treatment, we were able to reproduce the natural pattern of higher pupal masses of the directly developing generation. These data are therefore usable for the analysis of the proximate basis of attaining a size difference. We see that the larger final size of directly developing insects should be attributed to their higher growth rates but not to longer growing periods. This pattern differs from the emerging general picture according to which, when "needed," larger sizes of insects are attained through prolonging growth periods and not through increasing instantaneous growth rates. This applies to comparisons among sexes (Tammaru et al., 2010; Teder et al., 2014; Sõber et al., 2019), populations within species (Vellau et al., 2013; Meister et al., 2018), and also seasonal generations (Esperk et al., 2013). Our comparison of seasonal generations in *A. levana* broadens the view indicating that exceptions to this rule can exist. We propose that the situation in *A. levana* can be reconciled with the general picture assuming that here natural selection has targeted developmental periods specifically rather than body sizes. This view is corroborated by the much clearer and consistent among-generation differences in developmental periods than larval sizes as revealed by the present study. The longer development periods of the diapause development larvae are paralleled by similar observations in some other butterflies (Wiklund et al., 1991; Aalberg Haugen et al., 2012; Kivelä et al., 2019) and are predicted by some models (Kivelä et al., 2013). As the perhaps most intuitive explanation, such long developmental periods may be selected for to facilitate physiological preparation

for overwintering, whereas warning appearance and group living habits in *A. levana*—likely reducing larval mortality rates—may be seen as permissive for such a strategy to evolve. As an aside, the conclusion about flexibility of larval growth rates in *A. levana* is supported also by sex-related difference in this variable, detected in this study (c.f., Tammaru et al., 2010; Stillwell et al., 2014; Sõber et al., 2019).

It is notable that changes specific to developmental pathway were not limited to the final larval instar. Directly developing and overwintering insects differed in instar-specific growth periods, growth rates, and body sizes also in the penultimate instar. This result may be seen as corroborating the view that developmental constraints preclude considerable changes in larval growth parameters at the level of one larval instar (Tammaru, 1998; Kivelä et al., 2020), and any major differences have to be accumulated through modifying development during a number larval instars. This is, however, in some contrast with the observation that changes specific to developmental pathway are limited to the last larval instar in some other butterflies (Friberg et al., 2012; Kivelä et al., 2019).

In conclusion, the present study shows that controlled laboratory experiments comparing the growth schedules of different seasonal generations of insects can yield data usable for the analysis of seasonal adaptations, which largely provide the framework for life-history evolution in seasonal environments. Moreover, in a more general context, such comparisons yield information about what can and what cannot be plastically modified in the growth curve of an insect (Tammaru et al., 2015; Kivelä et al., 2020).

DATA AVAILABILITY STATEMENT

The data is now published and could be found here: https://doi.org/10.5061/dryad.xksn02vfh.

AUTHOR CONTRIBUTIONS

TE and TT contributed roughly equally to all stages of manuscript preparation. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.612330/full#supplementary-material

REFERENCES

Aalberg Haugen, I. M., Berger, D., and Gotthard, K. (2012). The evolution of alternative developmental pathways: footprints of selection on life-history traits in a butterfly. *J. Evol. Biol.* 25, 1377–1388. doi: 10.1111/j.1420-9101.2012.02525.x

Angilletta, M. J. Jr., Steury, T. D., and Sears, M. W. (2004). Temperature, growth rate, and body size in ectotherms: fitting pieces of a life-history puzzle. *Integr. Compar. Biol.* 44, 498–509. doi: 10.1093/icb/44.6.498

Arendt, J. D. (1997). Adaptive intrinsic growth rates: an integration across taxa. *Q. Rev. Biol.* 72, 149–177. doi: 10.1086/419764

Atkinson, D. (1994). Temperature and organism size *a* biological law for ectotherms. *Adv. Ecol. Res.* 25, 1–58. doi: 10.1016/S0065-2504(08)60212-3

Atkinson, D. (1996). "Ectotherm life-history responses to developmental temperature," in *Animals and Temperature: Phenotypic and Evolutionary Adaptation, Society for Experimental Biology Seminar Series*, eds I. Johnston and A. Bennett (Cambridge: Cambridge University Press), 183–204. doi: 10.1017/CBO9780511721854.009

Atkinson, D., and Sibly, R. M. (1997). Why are organisms usually bigger in colder environments? Making sense of a life history puzzle. *Trends Ecol. Evol.* 12, 235–239. doi: 10.1016/S0169-5347(97)01058-6

Baudach, A., Lee, K.-Z., Vogel, H., and Vilcinskas, A. (2018). Immunological larval polyphenism in the map butterfly *Araschnia levana* reveals the photoperiodic modulation of immunity. *Evol. Ecol.* 8, 4891–4898. doi: 10.1002/ece3.4047

Beck, S. D. (1980). *Insect Photoperiodism*. New York, NY: Academic Press.

Berger, D., Friberg, M., and Gotthard, K. (2011). Divergence and ontogenetic coupling of larval behaviour and thermal reaction norms in three closely related butterflies. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* 278, 313–320. doi: 10.1098/rspb.2010.1398

Berger, D., and Gotthard, K. (2008). Time stress, predation risk and diurnal-nocturnal foraging trade-offs in larval prey. *Behav. Ecol. Sociobiol.* 62, 1655–1663. doi: 10.1007/s00265-008-0594-4

Blanckenhorn, W. U. (2000). The evolution of body size: what keeps organisms small? *Q. Rev. Biol.* 75, 385–407. doi: 10.1086/393620

Chelini, M.-C., Delong, J. P., and Hebets, E. A. (2019). Ecophysiological determinants of sexual size dimorphism: integrating growth trajectories, environmental conditions, and metabolic rates. *Oecologia* 191, 61–71. doi: 10.1007/s00442-019-04488-9

Danks, H. V. (1987). *Insect Dormancy: an Ecological Perspective*. Ottawa: Biological Survey of Canada.

Dmitriew, C. M. (2011). The evolution of growth trajectories: what limits growth rate? *Biol. Rev.* 86, 97–116. doi: 10.1111/j.1469-185X.2010.00136.x

Esperk, T., Stefanescu, C., Teder, T., Wiklund, C., Kaasik, A., and Tammaru, T. (2013). Distinguishing between anticipatory and responsive plasticity in a seasonally polyphenic butterfly. *Evol. Ecol.* 27, 315–332. doi: 10.1007/s10682-012-9598-7

Esperk, T., and Tammaru, T. (2004). Does the 'investment principle' model explain moulting strategies in lepidopteran larvae? *Physiol. Entomol.* 29, 56–66. doi: 10.1111/j.1365-3032.2004.0365.x

Freitak, D., Tammaru, T., Sandre, S.-L., Meister, H., and Esperk, T. (2019). Longer life span is associated with elevated immune activity in a seasonally polyphenic butterfly. *J. Evol. Biol.* 32, 653–665. doi: 10.1111/jeb.13445

Friberg, M., Aalberg Haugen, I. M., Dahlerus, J., Gotthard, K., and Wiklund, C. (2011). Asymmetric life-history decision-making in butterfly larvae. *Oecologia* 165, 301–310. doi: 10.1007/s00442-010-1804-0

Friberg, M., Dahlerus, J., and Wiklund, C. (2012). Strategic larval decision-making in a bivoltine butterfly. *Oecologia* 169, 623–635. doi: 10.1007/s00442-011-2238-z

Friberg, M., and Karlsson, B. (2010). Life-history polyphenism in the map butterfly (*Araschnia levana*): developmental constraints versus season-specific adaptations. *Evol. Ecol. Res.* 12, 603–615.

Fric, Z., Klimova, M., and Konvička, M. (2006). Mechanical design indicates differences in mobility among butterfly generations. *Evol. Ecol. Res.* 8, 1511–1522.

Fric, Z., and Konvička, M. (2002). Generations of the polyphenic butterfly *Araschnia levana* differ in body design. *Evol. Ecol. Res.* 4, 1017–1032.

Hahn, D. A., and Denlinger, D. L. (2007). Meeting the energetic demands of insect diapause: nutrient storage and utilization. *J. Insect Physiol.* 53, 760–773. doi: 10.1016/j.jinsphys.2007.03.018

Hahn, D. A., and Denlinger, D. L. (2011). Energetics of insect diapause. *Annu. Rev. Entomol.* 56, 103–121. doi: 10.1146/annurev-ento-112408-085436

Ihalainen, E., and Lindstedt, C. (2012). Do avian predators select for seasonal polyphenism in the European map butterfly *Araschnia levana* (Lepidoptera: Nymphalidae)? *Biol. J. Linnean Soc.* 106, 737–748. doi: 10.1111/j.1095-8312.2012.01922.x

Kivelä, S. M., Davis, R. B., Esperk, T., Gotthard, K., Mutanen, M., Valdma, D., et al. (2020). Comparative analysis of larval growth in *Lepidoptera* reveals instar-level constraints. *Funct. Ecol.* 34, 1391–1403. doi: 10.1111/1365-2435.13556

Kivelä, S. M., Gotthard, K., and Lehmann, P. (2019). Developmental plasticity in metabolism but not in energy reserve accumulation in a seasonally polyphenic butterfly. *J. Exper. Biol.* 222:jeb202150. doi: 10.1242/jeb.202150

Kivelä, S. M., Svensson, B., Tiwe, A., and Gotthard, K. (2015). Thermal plasticity of growth and development varies adaptively among alternative developmental pathways. *Evolution* 69, 2399–2413. doi: 10.1111/evo.12734

Kivelä, S. M., Välimäki, P., and Gotthard, K. (2013). Seasonality maintains alternative life-history phenotypes. *Evolution* 67, 3145–3160. doi: 10.1111/evo.12181

Koštál, V. (2006). Eco-physiological phases of insect diapause. *J. Insect Physiol.* 52, 113–127. doi: 10.1016/j.jinsphys.2005.09.008

Leather, S. R., Walters, K. F. A., and Bale, J. S. (1993). *The Ecology of Insect Overwintering*. Cambridge: Cambridge University Press.

Lehmann, P., Pruisscher, P., Posledovich, D., Carlsson, M., Käkelä, R., Tang, P., et al. (2016). Energy and lipid metabolism during direct and diapause development in a pierid butterfly. *J. Exper. Biol.* 219, 3049–3060. doi: 10.1242/jeb.142687

Littell, R. C., Milliken, G. A., Stroup, W. W., Wolfinger, R. D., and Schabenberger, O. (2006). *SAS for Mixed Models*, 2nd Edn, Cary: SAS Institute.

Meister, H., Hämäläinen, R., Valdma, D., Martverk, M., and Tammaru, T. (2018). How to become larger: ontogenetic basis of among-population size differences in a moth. *Entomol. Exper. Appl.* 166, 4–16. doi: 10.1111/eea.12634

Meister, H., Esperk, T., Välimäki, P., and Tammaru, T. (2017a). Evaluating the role and measures of juvenile growth rate: latitudinal variation in insect life histories. *Oikos* 126, 1726–1737. doi: 10.1111/oik.04233

Meister, H., Tammaru, T., Sandre, S.-L., and Freitak, D. (2017b). Sources of variance in immunological traits: evidence of congruent latitudinal trends across species. *J. Exper. Biol.* 220, 2606–2615. doi: 10.1242/jeb.154310

Morehouse, N. I., Mandon, N., Christides, J. P., Body, M., Bimbard, G., and Casas, J. (2013). Seasonal selection and resource dynamics in a seasonally polyphenic butterfly. *J. Evol. Biol.* 26, 175–185. doi: 10.1111/jeb.12051

Nylin, S. (2013). Induction of diapause and seasonal morphs in butterflies and other insects: knowns, unknowns and the challenge of integration. *Physiol. Entomol.* 38, 96–104.

Reinhardt, R. (1984). *Der Landkärtchenfalter*, 2nd Edn, Wittenberg: A. Ziemsen Verlag.

Remmel, T., Davison, J., and Tammaru, T. (2011). Quantifying predation on folivorous insect larvae: the perspective of life-history evolution. *Biol. J. Linnean Soc.* 104, 1–18. doi: 10.1111/j.1095-8312.2011.01721.x

Remmel, T., and Tammaru, T. (2009). Size-dependent predation risk in tree-feeding insects with different colouration strategies: a field experiment. *J. Anim. Ecol.* 78, 973–980. doi: 10.1111/j.1365-2656.2009.01566.x

Remmel, T., Tammaru, T., and Mägi, M. (2009). Seasonal mortality trends in tree-feeding insects: a field experiment. *Ecol. Entomol.* 34, 98–106. doi: 10.1111/j.1365-2311.2008.01044.x

Roff, D. A. (1992). *The Evolution of Life Histories. Theory and Analysis*. New York, NY: Chapman and Hall.

Roff, D. A. (2002). *Life History Evolution*. Sunderland, MA: Sinauer Associates.

Ruf, C. (2002). *Social Life-Styles in Caterpillars: Behavioral Mechanisms and Ecological Consequences*. Bayreuth: Universität Bayreuth.

Saunders, D. S. (2020). Dormancy, diapause, and the role of the circadian system in insect photoperiodism. *Annu. Rev. Entomol.* 65, 373–389. doi: 10.1146/annurev-ento-011019-025116

Shapiro, A. M. (1976). Seasonal polyphenism. *Evol. Biol.* 9, 259–333.

Simpson, S. J., Sword, G. A., and Lo, N. (2011). Polyphenism in insects. *Curr. Biol.* 21, R738–R749. doi: 10.1016/j.cub.2011.06.006

Sõber, V., Sandre, S.-L., Esperk, T., Teder, T., and Tammaru, T. (2019). Ontogeny of sexual size dimorphism revisited: females grow for a longer time and also faster. *PLoS One* 14:e0215317. doi: 10.1371/journal.pone.0215317

Stearns, S. C. (1992). *The Evolution of Life Histories*. Oxford: Oxford University Press.

Stillwell, R. C., and Davidowitz, G. (2010). A developmental perspective on the evolution of sexual size dimorphism of a moth. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* 277, 2069–2074. doi: 10.1098/rspb.2009.2277

Stillwell, R. C., Daws, A., and Davidowitz, G. (2014). The ontogeny of sexual size dimorphism of a moth: when do males and females grow apart? *PLoS One* 9:e106548. doi: 10.1371/journal.pone.0106548

Tammaru, T. (1998). Determination of adult size in a folivorous moth: constraints at instar level? *Ecol. Entomol.* 23, 80–89. doi: 10.1046/j.1365-2311.1998.00106.x

Tammaru, T., and Esperk, T. (2007). Growth allometry of immature insects: larvae do not grow exponentially. *Funct. Ecol.* 21, 1099–1105. doi: 10.1111/j.1365-2435.2007.01319.x

Tammaru, T., Esperk, T., Ivanov, V., and Teder, T. (2010). Proximate sources of sexual size dimorphism in insects: locating constraints on larval growth schedules. *Evol. Ecol.* 24, 161–175. doi: 10.1007/s10682-009-9297-1

Tammaru, T., Vellau, H., Esperk, T., and Teder, T. (2015). Searching for constraints by cross-species comparison: reaction norms for age and size at maturity in insects. *Biol. J. Linnean Soc.* 114, 296–307. doi: 10.1111/bij.12417

Tauber, M. J., Tauber, C. A., and Masaki, S. (1986). *Seasonal Adaptations of Insects*. Oxford: Oxford University Press.

Teder, T., Esperk, T., Remmel, T., Sang, A., and Tammaru, T. (2010). Counterintuitive size patterns in bivoltine moths: late-season larvae grow larger despite lower food quality. *Oecologia* 162, 117–125. doi: 10.1007/s00442-009-1439-1

Teder, T., and Tammaru, T. (2005). Sexual size dimorphism within species increases with body size in insects. *Oikos* 108, 321–328. doi: 10.1111/j.0030-1299.2005.13609.x

Teder, T., Vellau, H., and Tammaru, T. (2014). Age and size at maturity: a quantitative review of diet-induced reaction norms in insects. *Evolution* 68, 3217–3228. doi: 10.1111/evo.12518

Tolman, T., and Lewington, R. (2008). *Collins Butterfly Guide. The Most Complete Guide to the Butterflies of Britain and Europe*. London: HarperCollins.

Vellau, H., Leppik, E., Frerot, B., and Tammaru, T. (2013). Detecting a difference in reaction norms for size and time at maturation: pheromone strains of the European corn borer (*Ostrinia nubilalis*: Lepidoptera: Crambidae). *Evol. Ecol. Res.* 15, 589–599.

Vendl, T., Šípek, P., Kouklík, O., and Kratochvíl, L. (2018). Hidden complexity in the ontogeny of sexual size dimorphism in male-larger beetles. *Nat. Sci. Rep.* 8:5871. doi: 10.1038/s41598-018-24047-1

Viidalepp, J., and Remm, H. (1996). *Eesti Liblikate Määraja*. Tallinn: Valgus.

Vilcinskas, A., and Vogel, H. (2016). Seasonal phenotype-specific transcriptional reprogramming during metamorphosis in the European map butterfly *Araschnia levana*. *Evol. Ecol.* 6, 3476–3485. doi: 10.1002/ece3.2120

Wang, X.-P., Yang, Q., Zhou, X.-M., Zhao, F., and Lei, C. (2007). Effect of photoperiod associated with diapause induction on the accumulation of metabolites in *Sericinus montelus* (Lepidoptera: Papilionidae). *Appl. Entomol. Zool.* 42, 419–424. doi: 10.1303/aez.2007.419

Whitman, D. W., and Agrawal, A. A. (2009). "What is phenotypic plasticity and why is it important?," in *Phenotypic Plasticity in Insects. Mechanisms and Consequences*, eds D. W. Whitman and T. N. Antakrishnan (Einfeld: Science Publishers), 1–63. doi: 10.1201/b10201

Wiklund, C., Nylin, S., and Forsberg, J. (1991). Sex-related variation in growth rate as a result of selection for large size and protandry in a bivoltine butterfly, *Pieris napi*. *Oikos* 60, 241–250.

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Exposure to Inactivated Deformed Wing Virus Leads to *Trans*-Generational Costs but Not Immune Priming in Honeybees (*Apis mellifera*)

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Pathogens are identified as one of the major drivers behind the honeybee colony losses, as well as one of the reasons for the reported declines in terrestrial insect abundances in recent decades. To fight infections, animals rely on their immune system. The immune system of many invertebrates can be primed by exposure to a pathogen, so that upon further exposure the animal is better protected. The protective priming effect can even extend to the next generation, but the species capable of priming the immune system of their offspring are still being investigated. Here we studied whether honeybees could prime their offspring against a viral pathogen, by challenging honeybee queens orally with an inactivated deformed wing virus (DWV), one of the most devastating honeybee viruses. The offspring were then infected by viral injection. The effects of immune priming were assayed by measuring viral loads and two typical symptoms of the virus, pupal mortality, and abnormal wing phenotype. We saw a low amount of wing deformities and low pupal mortality. While no clear priming effect against the virus was seen, we found that the maternal immune challenge, when combined with the stress caused by an injection during development, manifested in costs in the offspring, leading to an increased number of deformed wings.

Keywords: immune priming, DWV, *Apis mellifera*, *trans*-generational, costs

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INTRODUCTION

In their natural environment animals may encounter variety of stressors at any time. Among others, pathogens, parasites, and toxic substances have often significant negative fitness consequences, like reduced fecundity or survival (Kammenga et al., 1997; Fitze et al., 2004), especially when encountered simultaneously (Sih et al., 2004). Organisms can fight stressors by avoidance, physical barriers, or by physiological responses, for example by producing enzymes that break down harmful toxins (Feyereisen, 1999). The physiological resistance mechanism against pathogens is the immune system (Siva-Jothy et al., 2005). In vertebrates, adaptive immunity relies on production of antibodies, proteins that can recognize and quickly respond to the same pathogen later, providing long lasting protection (Farber et al., 2016). Despite lacking the immune machinery for producing

antibodies, insects, and other invertebrates are still able to prime their immune system and have increased protection against pathogens they have encountered before. An initial exposure to a pathogen primes the organisms innate immune system to provide increased protection upon secondary exposure, although the protection may vary in length and specificity (Milutinović and Kurtz, 2016). Immune priming has been observed in variety of invertebrate taxa, most notably in insect hosts, such as the Indian-meal moth (Tidbury et al., 2011), the red flour beetle (Roth et al., 2009), and the bumble bee (Sadd and Schmid-Hempel, 2006). The priming effects have been shown to extend to the next generation (Tetreau et al., 2019), providing offspring protection against pathogens that are expected to be found in the environment (Pigeault et al., 2016).

Understanding the interaction between stressors and resistance mechanisms is increasingly important, as more species are at risk of extinction (Bongaarts, 2019). In recent decades, alarming declines in insect populations have been observed (Forister et al., 2011; Hallmann et al., 2017). A new meta-analysis of available long term surveys from around the world estimated an 8.81% decline per decade in terrestrial insect abundance (Klink et al., 2020). The major drivers for this decline include habitat loss, climate change, stressors like synthetic pesticides and fertilizers, and pathogens (Sánchez-Bayo and Wyckhuys, 2019). Pathogens can seriously affect natural populations (Skerratt et al., 2007), but managed populations are suffering from disease outbreaks as well. Managed honeybees have also faced similar problems with bacterial and viral pathogens causing high colony mortality, especially when combined with other stressors like pesticides (Smith et al., 2013; Grassl et al., 2018), parasites (Nazzi et al., 2012), or poor nutrition (Belzunces et al., 2013). The increased mortality of honeybee colonies has received a lot of attention, as they are important pollinators of many crops (Klein et al., 2007). The diseases in managed honeybee colonies pose a risk to wild populations too, as viruses can spread between managed bees and wild bees when foraging in the same area (Fürst et al., 2014; Mazzei et al., 2014; Alger et al., 2019). One of the most devastating pathogens associated with the colony losses is the deformed wing virus (DWV), an RNA virus in the family Iflaviridae (de Miranda and Genersch, 2010). Stated symptoms of the DWV include bloated abdomen, discoloration, mortality at the pupal stage, and abnormally developed wings (de Miranda and Genersch, 2010). DWV is transmitted by the ectoparasitic mite *Varroa destructor*, which has spread all around the world, causing the ongoing pandemic among honeybees (Nazzi and Le Conte, 2016). Bees may get infected as adults by phoretic mites, or at the pupal stage in the enclosed brood cell. When in the brood cell, the mite feeds on the hemolymph and fat body of the developing bee, simultaneously infecting it with the virus (Ramsey et al., 2019).

A major antiviral defense mechanism in insects and many other organisms is the RNAi-system (Lemaitre and Hoffmann, 2007). Bees have a lower number of immune related genes when compared to most insects, but the same major pathways are functional, including the RNAi (Evans et al., 2006; Barribeau et al., 2015). The RNAi-system indeed seems to be involved in the honeybee antiviral defense (Brutscher and Flenniken, 2015). Studies done with DWV and Israeli Acute Paralysis Virus have shown that activating the RNAi-system by feeding virus-specific dsRNA to larvae or adult bees before an infection reduces the viral load, mortality and symptoms resulting from the specific viral infection (Hunter et al., 2010; Desai et al., 2012). The transfer of virus specific dsRNA or other virus derived immune elicitors to the next generation could therefore be a mechanism for *trans*-generational immune priming (TGIP). The mechanism for such maternal transfer of immune elicitors to the next generation has been discovered in honeybees (Salmela et al., 2015). After an immune challenge from bacteria, fragments of the bacteria bind to the multifunctional protein vitellogenin which is then transferred to the eggs and thus to the next generation (Salmela et al., 2015). Indeed, challenging the queen with a bacterial pathogen, *Paenibacillus larvae,* which is exclusively infecting only the young brood, increased resistance to this pathogen in the infected offspring (Hernández López et al., 2014). *Trans*-generational protection against viruses has been found in the nematode *Caenorhabditis elegans* (Rechavi et al., 2011) and in the Indian-meal moth *Plodia interpunctella* (Tidbury et al., 2011). Although observed in other hosts, the occurrence of antiviral TGIP in honeybees is unclear.

We used the honeybee-DWV system to investigate TGIP against viral pathogens. We hypothesized that a challenge of the honeybee queen with inactivated virus could lead to increased resistance against the virus in the offspring. We used heat inactivated DWV to orally challenge honeybee queens and infected the brood by DWV injection at the pupal stage. TGIP has been observed before with heat killed bacteria (Sadd et al., 2005; Hernández López et al., 2014), so we decided to use heat inactivated DWV to avoid exposing colonies to live pathogens which could spread to the environment. Injection resembles the natural infection route, as feeding mites also pierce the cuticle and viruses enter the hemolymph via saliva. Pupal mortality was measured following the infection. When the offspring emerged as adults, we measured the proportions of bees with wing deformities and viral loads with quantitative polymerase chain reaction (qPCR). In case the honeybee queen would be able to increase the resistance of their offspring against the DWV by TGIP, we would expect to see reduced symptoms or lower viral loads in the infected offspring.

MATERIALS AND METHODS

Honeybees and Virus

The honeybee (*Apis mellifera*) colonies used in this experiment were located in the Arizona State University Bee Lab in Mesa, AZ, United States. All colonies were healthy and had been subjected to 65% formic acid treatment to control Varroa. All queens were bred in the Bee lab and mated using open mating approach. After mating the queens were allowed to begin laying eggs in nucleus colony hive. Only sister queens were used for the study. DWV, clone-derived strain pDWV-USDA-703 of genotype DWV-A (Ryabov et al., 2019) was obtained as a ready suspension with concentration of 10^7 transcripts per µl from USDA Agricultural Research Center in Beltsville, MD, United States.

Queen Treatments

Twenty-four honeybee queens were divided into two treatment groups – a priming treatment and control treatment, 12 individuals in each. In the priming treatment the queens were orally immune challenged with inactivated DWV in feed (see below for details on feed preparation). Control queens were fed with Phosphate-buffered saline (PBS) in feed. After the queens were removed from their respective hives for the priming treatment, all the hives were treated with a queen mimicking pheromone to simulate the presence of the queen. Removed queens were placed in individual queen cages with the treatment queen feed and seven accompanying worker bees. The queen cages were kept in an incubator at 34°C on top of moistened sponges for 7 days. The queens were then returned to their respective hives in the queen cages, allowing the worker bees to free the queens via feeding though the remaining feed. To ensure that the brood used in the experiment was laid after the queens returned to the hives, a fresh frame was provided for egg laying.

Two queens died during the priming treatment (one from each treatment). The final number of queens returned to the hives was thus 11 per treatment, resulting in 22 queens in total.

Preparation of Queen Feed

The feed for queen treatments was prepared with inactivated virus suspended in a commercial bee feed containing 85.5% sugar (Ambrosia feed paste – Nordzucker, Germany). In total, 24 bee feed patties were prepared: 12 containing inactivated virus and 12 control patties. To inactivate the virus, the virus suspension was kept at 95°C for 60 min (MJ Research PTC-200 Peltier Thermal Cycler) (Carrillo-Tripp et al., 2016). Ninety-six microliters of inactivated DWV suspension and 454 μl of 1x PBS were mixed to obtain 550 μl of the DWV priming suspension. To prepare priming patties, 44 g of bee feed was quickly melted in a glass container in a microwave and mixed with 550 μl of the DWV priming suspension, resulting in 8 × 10^7 virus transcripts per patty. For control treatment patties, 550 μl of 1x PBS was mixed into the melted bee feed. The mixtures were poured into a thin aluminum foil vessel and let to solidify at 4°C.

Pupal Injection-Treatments

Following the queen treatments, when the newly laid brood reached the white-eyed pupal stage, 30 white-eyed pupae from each hive were extracted from the cells using forceps in the laboratory. After extraction, the pupae were subjected to one of three treatments, 10 pupae in each. As there were 11 hives in each queen treatment, and 10 pupae from each hive were subjected to one of the three pupal treatments, the number of pupae in the six different queen-pupae treatment combinations was 110. Two hives had only 28 right age pupae, and one hive had 29, making the total in these treatments 108 and 109, respectively (total $n = 655$). The pupae were injected either with 1 μl 1x PBS in the control treatment, 1 μl live 10^7 DWV suspension in the infection treatment or left untreated in the naïve treatment. The white-eyed stage is the stage at which the bees are naturally infected with the Varroa-mite (Donze and Guerin, 1994). One microliter of 10^7 DWV transcripts was chosen as the infection concentration, as a pre-experiment suggested that this concentration resulted in roughly equal amounts of good and deformed wings. Injections were performed using a 10 μl Hamilton syringe with a 30G needle (BD). The needle was inserted at a low angle between the fourth and fifth tergite in the lateral abdomen, close to the site on the body where the mite feeds (Rosenkranz et al., 2009; Boncristiani et al., 2013; Ramsey et al., 2019).

After the injections, the pupae were placed on a folded filter paper (**Supplementary Figure 1**) on a Petri dish and checked daily until emergence. The Petri dishes were kept on a water bath in a closed plastic container to ensure relative humidity over 50% (Williams et al., 2013). The plastic container was placed in an incubator at 34°C until the pupae emerged as adults.

Bioassays

Survival

Following the pupal injection treatments, survival of the pupae ($n = 655$) was monitored daily. After 10 days all individuals had either emerged as adult bees or died. Dead individuals were removed during monitoring.

Wing Phenotype

The wing phenotype was documented for each emerged adult bee ($n = 617$) at the day of emergence by visual observation. The wings were classified as being deformed or normal (**Supplementary Figure 2**).

Viral Load Quantification

A subset of three emerged adult bees of the ten pupae receiving injection treatment from each hive were used for the viral load assay (total $n = 185$). We chose to use only fat body as the sample to measure viral concentrations. DWV has been previously shown to be present in the abdomen of all infected bees in high numbers and specifically in the fat body (Yue and Genersch, 2005; Fievet et al., 2006), while contaminants from the eyes may inhibit PCR reactions (Evans et al., 2013). Viral loads were quantified by real-time quantitative PCR, and virus replication numbers were normalized to the reference gene *actin*.

RNA extraction

The worker bees were dissected, the fat body was placed in a 1.5 ml microcentrifuge tube and homogenized with a pestle in 300 μl of Trizol. Samples were stored overnight at −80°C, homogenized further, 700 μl of Trizol and 200 μl of chloroform per sample was added, mixed vigorously, and centrifuged at 12,000 *g* for 20 min at 4°C. The upper aqueous phase containing the RNA was then transferred to new tubes with 500 μl isopropyl alcohol and incubated in −80°C for 1 h. The samples were centrifuged again at 12,000 *g* for 30 min at 4°C. Supernatant was removed, and 1 ml of 75% ethanol was added to wash the RNA pellet. After a quick centrifugation, all supernatant was removed, and the samples were air dried for 10 min. The RNA pellet was then dissolved in 50 μl of nuclease free water and stored at −80°C until further analysis. To eliminate any genomic DNA contamination, all the RNA samples were treated with DNase, using DNase I, RNase-free kit (Thermo Fisher Scientific, United States)

according to manufacturer's instructions. RNA concentration was measured with NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, United States).

qPCR

Qiagen QuantiTect SYBR Green RT-PCR kit with 12.5 µl final reaction volume was used for the reverse transcription and qPCR reactions on a 384-well plate, with primers shown in **Supplementary Table 1**. The plates were ran on Bio-Rad C1000 Thermal Cycler CFX384 Real-Time System with an initial reverse transcription phase for 30 min at 50°C, an initial PCR activation step for 15 min at 95°C, followed by 40 cycles with 15 s of denaturation step at 94°C, 31 s annealing step at 52°C and 30 s extension and data collection step at 72°C.

Statistical Methods

All analyses were done with R version 3.4.1 (R Core Team, 2017). For statistical analyses packages lme4 (Bates et al., 2015), survival (Therneau, 2015), multcomp (Hothorn et al., 2008), and nlme (Pinheiro et al., 2021) were used, and for figures survminer (Kassambara and Kosinski, 2017).

Pupal survival was analyzed with survival regression analysis. Queen treatment and pupal treatment were included as fixed effects, as well as interaction. Hive was treated as a random effect in the model.

Interactions between the treatment groups for wing phenotypes were compared with a generalized linear mixed model (Bolker et al., 2009), with binomial distribution and probit link function. Wing phenotype was the response variable, queen and pupal treatments were included as fixed effects, with interaction, and hive as a random effect.

Viral loads between groups were analyzed by calculating the ΔCt according to the 2-ΔΔCt-method (Livak and Schmittgen, 2001). We used primers that have been used in several studies, with reported efficiencies close to 2 (**Supplementary Table 1**). Viral loads were analyzed with a weighted linear mixed model, log-transformed 2^-ΔCt-value being the response variable and queen and pupal treatments were the interacting fixed effects, with hive as a random effect. The individuals from one control queen plus three other individuals were excluded from this analysis because there was not enough RNA in the samples, and one outlier was removed. Calculated dCt values are available in **Supplementary Table 2**.

RESULTS

Pupal Survival

After the pupal treatments, the mortality of the pupae was documented. Mortality was below 11% in all groups, even in the ones infected with virus (**Figure 1**). Pupal mortality was not affected by the queen priming treatments or injection of the virus, as there were no significant effects in the survival model (**Table 1**).

Wing Phenotype

We observed the wing phenotype of each emerged adult bee and found deformed wings in all treatment groups, also in the ones not infected with the virus (**Figure 2**). The percentage of normally developed wings in the infected pupae from primed and non-primed queens were 62% and 60%, while in the naïve group they were 72% and 70%, respectively. In the PBS-injected group 74% of the offspring of non-primed queens had normally developed wings, but only 58% of the offspring of primed queens. The interaction of queen treatment and PSB-injection treatment thus caused an increase in the number of wing deformities (**Figure 2** and **Table 2**). Otherwise no significant effects were found.

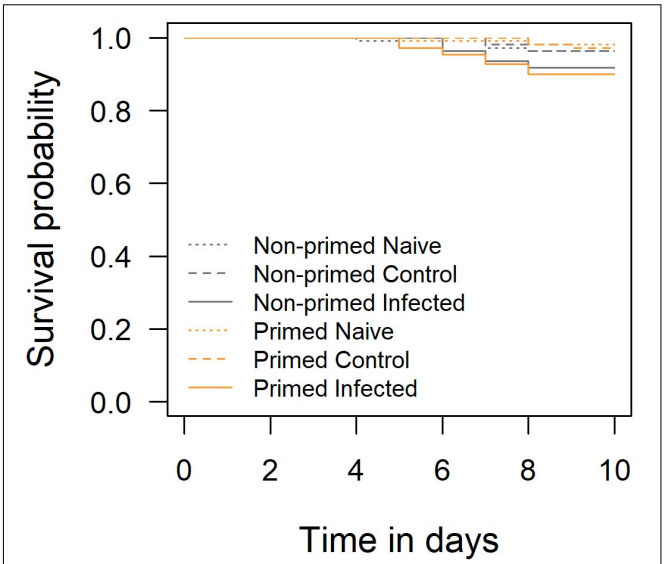

FIGURE 1 | Survival probabilities of pupae after pupal injection treatments. Orange lines showing pupae from primed queens, gray lines from non-primed control queens.

TABLE 1 | Results from survival regression model.

Parameter	Estimate	SE	*z*	*P*-value
Intercept	**3.503**	**0.288**	**12.14**	**<0.0001**
Queen primed	0.283	0.302	0.94	0.35
Pupae control	0.005	0.242	0.02	0.98
Pupae infected	−0.294	0.210	−1.40	0.16
Queen primed × Pupae control	−0.142	0.395	−0.36	0.72
Queen primed × Pupae infected	−0.315	0.337	−0.93	0.35

Bold was used to highlight significant values ($p < 0.05$).

Viral Load

To measure viral load the relative loads were quantified by real time quantitative PCR. The highest relative viral loads were observed in the infected pupae of primed and non-primed queens (**Figure 3** and **Table 3**). Naïve and control pupae had lower but detectable viral loads, even though not infected in the experiment. The lowest viral loads were found in the naïve offspring of non-primed queens, while the PBS-injected offspring from non-primed queens had slightly higher viral load. The PBS-injected offspring of primed queens had load

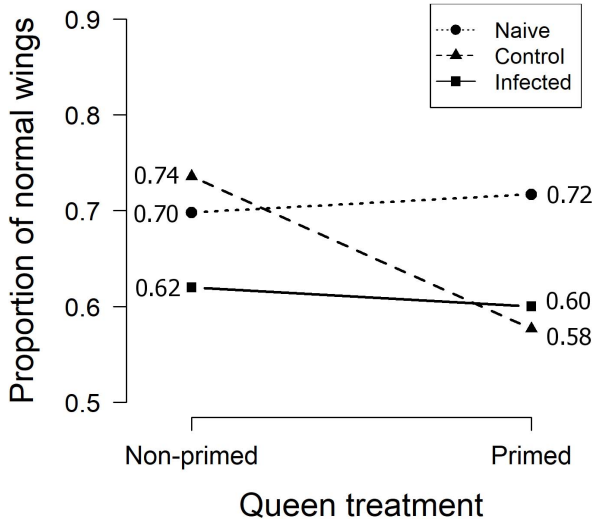

FIGURE 2 | Proportions of normally developed wings among emerged adult bees of non-primed and primed queens. *Y*-axis from 0.5 to 0.9.

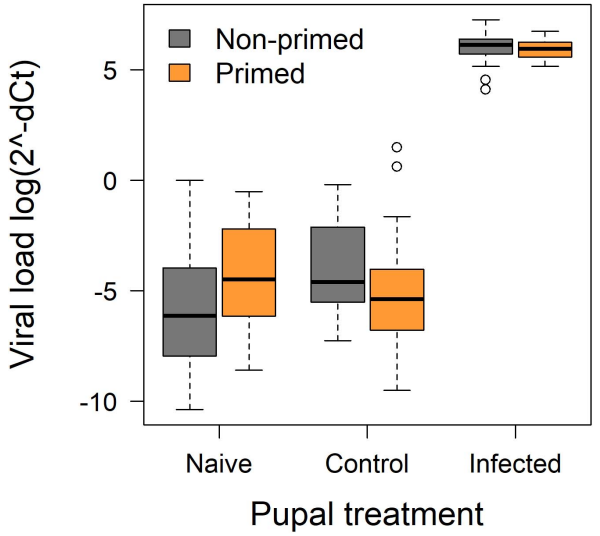

FIGURE 3 | Median and quartiles of the log(2^−ΔCt)-values for each treatment group with outliers. Orange boxes represent offspring from primed queens, gray from non-primed control queens.

TABLE 2 | Results from the generalized linear mixed model on wing phenotype.

Parameter	Estimate	SE	*Z*	*P*-value
Intercept	**0.525**	**0.153**	**3.416**	**0.0006**
Queen primed	0.076	0.219	0.346	0.729
Pupae control	0.131	0.186	0.706	0.480
Pupae infected	−0.222	0.184	−1.209	0.227
Queen primed × Pupae control	**−0.537**	**0.262**	**−2.055**	**0.039**
Queen primed × Pupae infected	−0.120	0.262	−0.456	0.648

Bold was used to highlight significant values ($p < 0.05$).

TABLE 3 | Results from the weighted linear mixed model on viral loads.

Parameter	Estimate	SE	*t*	*P*-value
Intercept	**−5.775**	**0.477**	**−12.117**	**<0.001**
Queen primed	**1.408**	**0.629**	**2.238**	**0.037**
Pupae control	**1.722**	**0.599**	**2.876**	**0.005**
Pupae infected	**11.811**	**0.477**	**24.761**	**<0.001**
Queen primed × Pupae control	**−2.803**	**0.863**	**−3.246**	**0.001**
Queen primed × Pupae infected	**−1.515**	**0.627**	**−2.419**	**0.017**

Bold was used to highlight significant values ($p < 0.05$).

similar to the naïve baseline. All the terms in the model were significant (**Table 3**).

DISCUSSION

In this study we see pronounced effect of maternal stressors on the wing deformation of the offspring and complex effects on the offspring viral load. At the same time, no clear priming effect of challenging the queens with heat inactivated DWV was evident in the offspring. The pupal mortality was low in both infected groups, and no differences in mortality was found between the infected and control pupae. This is consistent with other studies (Möckel et al., 2011; Remnant et al., 2019; Tehel et al., 2019), showing that DWV infection alone might not result in high pupal mortality.

The PBS-injected offspring of primed queens had significantly more deformed wings than the offspring of non-primed queens, indicating a *trans*-generational cost from the maternal immune challenge. We do not see as many deformed wings in the primed naïve offspring, indicating that the cost of the queen immune challenge is only evident in the offspring when they were also exposed to another stressor, the injection treatment. The injection causes wounding, which may cause unspecific immune system activation and additional costs (Erler et al., 2011). It is possible that all offspring of primed queens suffered costs in other aspects that were not investigated in this experiment. Some wing deformities in all treatments were probably caused by handling stress, as artificial rearing conditions in the lab are suboptimal for bee development. Our results showing costs from maternal immune challenge are generally consistent with previous TGIP studies showing developmental costs for the offspring. We know that maternal immune challenges can cause tradeoffs in the offspring, like prolonged developmental time that were observed in the beetles *Tenebrio molitor* and *Tribolium castaneum* (Zanchi et al., 2011; Schulz et al., 2019). In the moth *Manduca sexta* the individuals developed faster, but had reduced fecundity (Trauer and Hilker, 2013). Costs from maternal immune challenge for offspring may also manifest in other ways, as was shown in bumble bees, when maternal immune challenge reduced offspring resistance to unrelated pathogens (Sadd and Schmid-Hempel, 2009).

Previously wing deformities have been associated with the horizontal transmission by Varroa mite feeding, as similar injection experiments have resulted in wing deformities in a dose dependent manner (Möckel et al., 2011). Also, the DWV genotype A has been claimed less virulent than genotype B (McMahon et al., 2016). In this study using DWV genotype A, we observed both low mortality as well as low amount of wing deformities, when compared to other studies (Tehel et al., 2019; Dubois et al., 2020). We saw deformed wings in only 38% of the primed and 40% of the non-primed infected bees, while in the non-primed control group 24% had deformed wings. In other studies with similar methodology, a substantially lower inoculation concentration lead to 60–74% of bees developing deformed wings, while the number in control groups were similar to ours (Tehel et al., 2019; Dubois et al., 2020). However, these studies used inocula extracted from naturally infected bees, whereas an inoculum prepared from honeybee pupae infected with a cDNA clone was used in our study (Ryabov et al., 2019). Our results indicate that even after infected with a high concentration of DWV (10^7/μl), pupal mortality and amount of wing deformities may still be low relative to control, probably because of low virulence of the particular strain.

We found high but similar viral loads in the infected groups, regardless of the maternal priming treatment. The lowest viral load was found in the naïve offspring of non-primed queens. DWV is often found even in asymptomatic hives as a covert infection (Martin and Brettell, 2019), and in our case the virus is indeed detectable as a background baseline infection in the bees that were not injected with the virus. Interestingly, we found higher viral titers in naïve individuals from primed queens. This could result from the transmission of viral templates from the queen priming treatment that were then detected by the real-time quantitative PCR, consistent with the transfer of pathogen particles as a mechanism for TGIP (Salmela et al., 2015). The PBS-injected offspring from non-primed queens had slightly higher viral load, showing that the injection of PBS alone could lead to the higher titers of a latent background infection, as has been shown before (Anderson and Gibbs, 1988). In contrast, the PBS-injected offspring of primed queens had comparably lower viral load, similar to the naïve baseline. We hypothesize, that the background infection is low enough to not induce an antiviral response (Moreno-García et al., 2014), but the additional challenges of injection and maternal immune challenge caused an increased immune response, activating also the antiviral pathways. This resulted in the observed lower viral titers in the primed control bees, but immune responses being costly (Moret and Schmid-Hempel, 2000), contributed to the increased wing deformities observed in this treatment group.

Although TGIP with viruses have been studied less, recently the existence of antiviral TGIP in fruit flies and mosquitoes was shown, lasting for multiple generations (Mondotte et al., 2020). The mechanism was found to be virus specific, RNAi independent and was shown with multiple positive strand RNA-viruses, which the DWV also is. In mosquitoes the oral infection route was also effective, although live virus was used. The methods in our study are closely resembling the ones in the mosquito experiment, hence similar effect on offspring could be expected. However, it is not certain whether using inactivated virus for queen exposure affects TGIP, as there are no TGIP studies comparing the efficacy of inactivated versus live viruses (Tetreau et al., 2019). TGIP with killed bacteria has been shown in several systems (Sadd et al., 2005; Yue et al., 2013; Hernández López et al., 2014; Dubuffet et al., 2015; Tate and Graham, 2015; Rosengaus et al., 2017), but with fungal pathogens TGIP was only achieved with live fungi (Fisher and Hajek, 2015; Bordoni et al., 2018). Further, if antiviral TGIP can persist for multiple generations, exposure to DWV in prior generations may have already primed the queens, explaining the lack of further priming effects. Future studies could investigate whether viral derived DNA fragments are present in offspring of honeybee queens challenged with live virus or viral dsRNA, as was seen in the fruit flies by Mondotte et al. (2020).

In our experiment we would have expected to see a lower viral titer and reduced number of deformed wings in the primed group that was infected with the virus, if TGIP had clear beneficial effects for honeybees against viruses. Instead, we saw no reduction in symptoms and complex effects on viral loads. Contrasting results have been found in the nematode host *Caenorhabditis elegans*, where *trans*-generational effects were shown to cause viral silencing for many generations (Rechavi et al., 2011). In the moth *Plodia interpunctella trans*-generational protection against a DNA-virus was seen as lower susceptibility, although viral loads were not measured (Tidbury et al., 2011). The lack of clear TGIP effects in our system could be linked to the low virulence of the particular viral strain used, as typical DWV symptoms were marginal. Still, lack of clear beneficial TGIP effects against a viral pathogen is noteworthy, as the occurrence of and mechanisms TGIP is still being investigated (Tetreau et al., 2019). Theoretically TGIP is predicted when offspring dispersal is low and the pathogen threat is similar between generations (Pigeault et al., 2016). The honeybee offspring live in the same colony as the queen and multiple offspring generation overlap, suggesting that TGIP in this host should be very beneficial.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

DF, ML, and GA contributed to conception, design, and execution of the study. GA provided research materials and facilities. ML wrote the first draft of the manuscript. All authors contributed to revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.626670/full#supplementary-material

Supplementary Figure 1 | Pupae on folded filter paper in a Petri dish after injection.

Supplementary Figure 2 | Newly emerged honeybees, when the wing phenotype can be observed for the first time. Example of a honeybee with deformed wings **(A)** and with properly developed wings, both pairs of wings visible **(B)**.

REFERENCES

Alger, S. A., Burnham, P. A., Boncristiani, H. F., and Brody, A. K. (2019). RNA virus spillover from managed honeybees (*Apis mellifera*) to wild bumblebees (*Bombus spp.*). *PLoS One* 14:e0217822. doi: 10.1371/journal.pone.0217822

Anderson, D. L., and Gibbs, A. J. (1988). Inapparent Virus Infections and their Interactions in Pupae of the Honey Bee (*Apis mellifera Linnaeus*) in Australia. *J. Gen. Virol.* 69, 1617–1625. doi: 10.1099/0022-1317-69-7-1617

Barribeau, S. M., Sadd, B. M., du Plessis, L., Brown, M. J. F., Buechel, S. D., Cappelle, K., et al. (2015). A depauperate immune repertoire precedes evolution of sociality in bees. *Genome Biol.* 16:83. doi: 10.1186/s13059-015-0628-y

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* 67:i01. doi: 10.18637/jss.v067.i01

Belzunces, L. P., Kretzschmar, A., Salignon, M., Le Conte, Y., Brunet, J.-L., Suchail, S., et al. (2013). Influence of Pollen Nutrition on Honey Bee Health: Do Pollen Quality and Diversity Matter? *PLoS One* 8:e72016. doi: 10.1371/journal.pone.0072016

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H., et al. (2009). Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Ecol. Evol.* 24, 127–135. doi: 10.1016/j.tree.2008.10.008

Boncristiani, H. F., Evans, J. D., Chen, Y., Pettis, J., Murphy, C., Lopez, D. L., et al. (2013). In Vitro Infection of Pupae with Israeli Acute Paralysis Virus Suggests Disturbance of Transcriptional Homeostasis in Honey Bees (*Apis mellifera*). *PLoS One* 8:e73429. doi: 10.1371/journal.pone.0073429

Bongaarts, J. (2019). IPBES, 2019. Summary for policymakers of the global assessment report on biodiversity and ecosystem services of the Intergovernmental Science—Policy Platform on Biodiversity and Ecosystem Services. *Popul. Dev. Rev.* 45, 680–681. doi: 10.1111/padr.12283

Bordoni, A., Dapporto, L., Tatini, I., Celli, M., Bercigli, M., Barrufet, S. R., et al. (2018). Trans-generational immunization in the acrobat ant *Crematogaster scutellaris*. *Biol. Lett.* 14:20170761. doi: 10.1098/rsbl.2017.0761

Brutscher, L. M., and Flenniken, M. L. (2015). RNAi and antiviral defense in the honey bee. *J. Immunol. Res.* 2015:941897. doi: 10.1155/2015/941897

Carrillo-Tripp, J., Dolezal, A. G., Goblirsch, M. J., Miller, W. A., Toth, A. L., and Bonning, B. C. (2016). In vivo and in vitro infection dynamics of honey bee viruses. *Sci. Rep.* 6:22265. doi: 10.1038/srep22265

de Miranda, J. R., and Genersch, E. (2010). Deformed wing virus. *J. Invertebr. Pathol.* 103, S48–S61. doi: 10.1016/j.jip.2009.06.012

Desai, S. D., Eu, Y.-J., Whyard, S., and Currie, R. W. (2012). Reduction in deformed wing virus infection in larval and adult honey bees (*Apis mellifera L.*) by double-stranded RNA ingestion. *Insect Mol. Biol.* 21, 446–455. doi: 10.1111/j.1365-2583.2012.01150.x

Donze, G., and Guerin, P. M. (1994). Behavioral atributes and parental care of Varroa mites parasitizing honey bee brood. *Behav. Ecol. Sociobiol.* 34, 305–319. doi: 10.1007/BF00197001

Dubois, E., Dardouri, M., Schurr, F., Cougoule, N., Sircoulomb, F., and Thiéry, R. (2020). Outcomes of honeybee pupae inoculated with deformed wing virus genotypes A and B. *Apidologie* 51, 18–34. doi: 10.1007/s13592-019-00701-z

Dubuffet, A., Zanchi, C., Boutet, G., Moreau, J., Teixeira, M., and Moret, Y. (2015). Trans-generational Immune Priming Protects the Eggs Only against Gram-Positive Bacteria in the Mealworm Beetle. *PLoS Pathog.* 11:e1005178. doi: 10.1371/journal.ppat.1005178

Erler, S., Popp, M., and Lattorff, H. M. G. (2011). Dynamics of Immune System Gene Expression upon Bacterial Challenge and Wounding in a Social Insect (*Bombus terrestris*). *PLoS One* 6:e18126. doi: 10.1371/journal.pone.0018126

Evans, J. D., Aronstein, K., Chen, Y. P., Hetru, C., Imler, J. L., Jiang, H., et al. (2006). Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Mol. Biol.* 15, 645–656. doi: 10.1111/j.1365-2583.2006.00682.x

Evans, J. D., Schwarz, R. S., Ping Chen, Y., Budge, G., Cornman, R. S., De la Rua, P., et al. (2013). Standard methods for molecular research in *Apis mellifera*. *J. Apic. Res.* 52, 1–54. doi: 10.3896/IBRA.1.52.4.11

Farber, D. L., Netea, M. G., Radbruch, A., Rajewsky, K., and Zinkernagel, R. M. (2016). Immunological memory: Lessons from the past and a look to the future. *Nat. Rev. Immunol.* 16, 124–128. doi: 10.1038/nri.2016.13

Feyereisen, R. (1999). Insect P450 enzymes. *Annu. Rev. Entomol.* 44, 507–533. doi: 10.1146/annurev.ento.44.1.507

Fievet, J., Tentcheva, D., Gauthier, L., De Miranda, J., Cousserans, F., Colin, M. E., et al. (2006). Localization of deformed wing virus infection in queen and drone *Apis mellifera L. Virol. J.* 3:16. doi: 10.1186/1743-422X-3-16

Fisher, J. J., and Hajek, A. E. (2015). Maternal exposure of a beetle to pathogens protects offspring against fungal disease. *PLoS One* 10:e0125197. doi: 10.1371/journal.pone.0125197

Fitze, P. S., Tschirren, B., and Richner, H. (2004). Life history and fitness consequences of ectoparasites. *J. Anim. Ecol.* 73, 216–226. doi: 10.1111/j.0021-8790.2004.00799.x

Forister, M. L., Jahner, J. P., Casner, K. L., Wilson, J. S., and Shapiro, A. M. (2011). The race is not to the swift: Long-term data reveal pervasive declines in California's low-elevation butterfly fauna. *Ecology* 92, 2222–2235. doi: 10.1890/11-0382.1

Fürst, M. A., McMahon, D. P., Osborne, J. L., Paxton, R. J., and Brown, M. J. F. (2014). Disease associations between honeybees and bumblebees as a threat to wild pollinators. *Nature* 506, 364–366. doi: 10.1038/nature12977

Grassl, J., Holt, S., Cremen, N., Peso, M., Hahne, D., and Baer, B. (2018). Synergistic effects of pathogen and pesticide exposure on honey bee (*Apis mellifera*) survival and immunity. *J. Invertebr. Pathol.* 159, 78–86. doi: 10.1016/j.jip.2018.10.005

Hallmann, C. A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., et al. (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS One* 12:e0185809. doi: 10.1371/journal.pone.0185809

Hernández López, J., Schuehly, W., Crailsheim, K., and Riessberger-Gallé, U. (2014). Trans-generational immune priming in honeybees. *Proc. R. Soc. B* 281:20140454. doi: 10.1098/rspb.2014.0454

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous Inference in Genneral Parametric Models. *Biometr. J.* 50, 346–363. doi: 10.1002/bimj.200810425

Hunter, W., Ellis, J., Vanengelsdorp, D., Hayes, J., Westervelt, D., Glick, E., et al. (2010). Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (*Apis mellifera*, hymenoptera: Apidae). *PLoS Pathog.* 6:1001160. doi: 10.1371/journal.ppat.1001160

Kammenga, J. E., Van Koert, P. H. G., Koeman, J. H., and Bakker, J. (1997). Fitness consequences of toxic stress evaluated within the context of phenotypic plasticity. *Ecol. Appl.* 7, 726–734. doi: 10.1890/1051-07611997007[0726:FCOTSE]2.0.CO;2

Kassambara, A., and Kosinski, M. (2017). *survminer: Drawing Survival Curves using "ggplot2"*. Vienna: R Core Team.

Klein, A.-M., Vaissiere, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., et al. (2007). Importance of pollinators in changing landscapes for world crops. *Proc. R. Soc. B.* 274, 303–313. doi: 10.1098/rspb.2006.3721

Klink, R., van, Bowler, D. E., Gongalsky, K. B., Swengel, A. B., Gentile, A., et al. (2020). Meta-analysis reveals declines in terrestrial but increases in freshwater insect abundances. *Science* 368, 417–420. doi: 10.1126/SCIENCE.AAX9931

Lemaitre, B., and Hoffmann, J. (2007). The Host Defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.* 25, 697–743. doi: 10.1146/annurev.immunol.25.022106.141615

Leponiemi, M. (2018). *Vaccination of honey bees against Deformed Wing Virus*. Ph. D. thesis, Helsinki: University of Helsinki.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Martin, S. J., and Brettell, L. E. (2019). Deformed Wing Virus in Honeybees and Other Insects. *Annu. Rev. Virol.* 6, 49–69. doi: 10.1146/annurev-virology-092818-015700

Mazzei, M., Carrozza, M. L., Luisi, E., Forzan, M., Giusti, M., Sagona, S., et al. (2014). Infectivity of DWV associated to flower pollen: Experimental evidence of a horizontal transmission route. *PLoS One* 9:e113448. doi: 10.1371/journal.pone.0113448

McMahon, D. P., Natsopoulou, M. E., Doublet, V., Fürst, M., Weging, S., Brown, M. J. F., et al. (2016). Elevated virulence of an emerging viral genotype as a driver of honeybee loss. *Proc. R. Soc. B* 283:20160811. doi: 10.1098/rspb.2016.0811

Milutinović, B., and Kurtz, J. (2016). Immune memory in invertebrates. *Semin. Immunol.* 28, 328–342. doi: 10.1016/j.smim.2016.05.004

Möckel, N., Gisder, S., and Genersch, E. (2011). Horizontal transmission of deformed wing virus: Pathological consequences in adult bees (*Apis mellifera*) depend on the transmission route. *J. Gen. Virol.* 92, 370–377. doi: 10.1099/vir.0.025940-0

Mondotte, J. A., Gausson, V., Frangeul, L., Suzuki, Y., Vazeille, M., Mongelli, V., et al. (2020). Evidence For Long-Lasting Transgenerational Antiviral Immunity in Insects. *Cell Rep.* 33:108506. doi: 10.1016/j.celrep.2020.108506

Moreno-García, M., Condé, R., Bello-Bedoy, R., and Lanz-Mendoza, H. (2014). The damage threshold hypothesis and the immune strategies of insects. *Infect. Genet. Evol.* 24, 25–33. doi: 10.1016/j.meegid.2014.02.010

Moret, Y., and Schmid-Hempel, P. (2000). Survival for immunity: The price of immune system activation for bumblebee workers. *Science* 290, 1166–1168. doi: 10.1126/science.290.5494.1166

Nazzi, F., and Le Conte, Y. (2016). Ecology of *Varroa destructor*, the Major Ectoparasite of the Western Honey Bee, *Apis mellifera*. *Annu. Rev. Entomol.* 61, 417–432. doi: 10.1146/annurev-ento-010715-023731

Nazzi, F., Brown, S. P., Annoscia, D., Del Piccolo, F., Di Prisco, G., Varricchio, P., et al. (2012). Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. *PLoS Pathog.* 8:e1002735. doi: 10.1371/journal.ppat.1002735

Pigeault, R., Garnier, R., Rivero, A., and Gandon, S. (2016). Evolution of transgenerational immunity in invertebrates. *Proc. R. Soc. B* 283:20161136. doi: 10.1098/rspb.2016.1136

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and Team, R. C. (2021). *Linear and Nonlinear Mixed Effects Models*. Vienna: R Core Team.

R Core Team (2017). *R: A language and environment for statistical computing*. Vienna: R Core Team.

Ramsey, S. D., Ochoa, R., Bauchan, G., Gulbronson, C., Mowery, J. D., Cohen, A., et al. (2019). *Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph. *Proc. Natl. Acad. Sci. U S A.* 116, 1792–1801. doi: 10.1073/pnas.1818371116

Rechavi, O., Minevich, G., and Hobert, O. (2011). Transgenerational inheritance of an acquired small RNA-based antiviral response in *C. elegans*. *Cell* 147, 1248–1256. doi: 10.1016/j.cell.2011.10.042

Remnant, E. J., Mather, N., Gillard, T. L., Yagound, B., and Beekman, M. (2019). Direct transmission by injection affects competition among RNA viruses in honeybees. *Proc. R. Soc. B* 286:20182452. doi: 10.1098/rspb.2018.2452

Rosengaus, R. B., Hays, N., Biro, C., Kemos, J., Zaman, M., Murray, J., et al. (2017). Pathogen-induced maternal effects result in enhanced immune responsiveness across generations. *Ecol. Evol.* 7, 2925–2935. doi: 10.1002/ece3.2887

Rosenkranz, P., Aumeier, P., and Ziegelmann, B. (2009). Biology and control of *Varroa destructor*. *J. Invertebr. Pathol.* 103, S96–S119. doi: 10.1016/j.jip.2009.07.016

Roth, O., Sadd, B. M., Schmid-Hempel, P., and Kurtz, J. (2009). Strain-specific priming of resistance in the red flour beetle, *Tribolium castaneum*. *Proc. R. Soc. B* 276, 145–151. doi: 10.1098/rspb.2008.1157

Ryabov, E. V., Childers, A. K., Lopez, D., Grubbs, K., Posada-Florez, F., Weaver, D., et al. (2019). Dynamic evolution in the key honey bee pathogen deformed wing virus: Novel insights into virulence and competition using reverse genetics. *PLoS Biol.* 17:e3000502. doi: 10.1371/journal.pbio.3000502

Sadd, B. M., and Schmid-Hempel, P. (2006). Insect Immunity Shows Specificity in Protection upon Secondary Pathogen Exposure. *Curr. Biol.* 16, 1206–1210. doi: 10.1016/j.cub.2006.04.047

Sadd, B. M., and Schmid-Hempel, P. (2009). A distinct infection cost associated with trans-generational priming of antibacterial immunity in bumble-bees. *Biol. Lett.* 5, 798–801. doi: 10.1098/rsbl.2009.0458

Sadd, B. M., Kleinlogel, Y., Schmid-Hempel, R., and Schmid-Hempel, P. (2005). Trans-generational immune priming in a social insect. *Biol. Lett.* 1, 386–388. doi: 10.1098/rsbl.2005.0369

Salmela, H., Amdam, G. V., and Freitak, D. (2015). Transfer of Immunity from Mother to Offspring Is Mediated via Egg-Yolk Protein Vitellogenin. *PLoS Pathog.* 11:e1005015. doi: 10.1371/journal.ppat.1005015

Sánchez-Bayo, F., and Wyckhuys, K. A. G. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biol. Conserv.* 232, 8–27. doi: 10.1016/j.biocon.2019.01.020

Schulz, N. K. E., Sell, M. P., Ferro, K., Kleinhölting, N., and Kurtz, J. (2019). Transgenerational developmental effects of immune priming in the red flour beetle *Tribolium castaneum*. *Front. Physiol.* 10:98. doi: 10.3389/fphys.2019.00098

Sih, A., Bell, A. M., and Kerby, J. L. (2004). Two stressors are far deadlier than one. *Trends Ecol. Evol.* 19, 274–276. doi: 10.1016/j.tree.2004.02.010

Siva-Jothy, M. T., Moret, Y., and Rolff, J. (2005). Insect Immunity: An Evolutionary Ecology Perspective. *Adv. Insect Phys.* 32, 1–48. doi: 10.1016/S0065-2806(05)32001-7

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillott, A. D., et al. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth* 4, 125–134. doi: 10.1007/s10393-007-0093-5

Smith, K. M., Loh, E. H., Rostal, M. K., Zambrana-Torrelio, C. M., Mendiola, L., and Daszak, P. (2013). Pathogens, pests, and economics: Drivers of honey bee colony declines and losses. *Ecohealth* 10, 434–445. doi: 10.1007/s10393-013-0870-2

Tate, A. T., and Graham, A. L. (2015). Trans−generational priming of resistance in wild flour beetles reflects the primed phenotypes of laboratory populations and is inhibited by co−infection with a common parasite. *Funct. Ecol.* 29, 1059–1069. doi: 10.1111/1365-2435.12411

Tehel, A., Vu, Q., Bigot, D., Gogol-Döring, A., Koch, P., Jenkins, C., et al. (2019). The Two Prevalent Genotypes of an Emerging Infectious Disease, Deformed Wing Virus, Cause Equally Low Pupal Mortality and Equally High Wing Deformities in Host Honey Bees. *Viruses* 11:114. doi: 10.3390/v11020114

Tetreau, G., Dhinaut, J., Gourbal, B., and Moret, Y. (2019). Trans-generational immune priming in invertebrates: Current knowledge and future prospects. *Front. Immunol.* 10:1938. doi: 10.3389/fimmu.2019.01938

Therneau, T. M. (2015). *A Package for Survival Analysis in R. version 2.38*. Vienna: R Core Team.

Tidbury, H. J., Pedersen, A. B., and Boots, M. (2011). Within and transgenerational immune priming in an insect to a DNA virus. *Proc. R. Soc. B* 278, 871–876. doi: 10.1098/rspb.2010.1517

Trauer, U., and Hilker, M. (2013). Parental Legacy in Insects: Variation of Transgenerational Immune Priming during Offspring Development. *PLoS One* 8:e63392. doi: 10.1371/journal.pone.0063392

Williams, G. R., Alaux, C., Costa, C., Csáki, T., Doublet, V., Eisenhardt, D., et al. (2013). Standard methods for maintaining adult *Apis mellifera* in cages

under in vitro laboratory conditions. *J. Apic. Res.* 52, 1–36. doi: 10.3896/IBRA.1.52.1.04

Yue, C., and Genersch, E. (2005). RT-PCR analysis of Deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). *J. Gen. Virol.* 86, 3419–3424. doi: 10.1099/vir.0.81401-0

Yue, F., Zhou, Z., Wang, L., Ma, Z., Wang, J., Wang, M., et al. (2013). Maternal transfer of immunity in scallop *Chlamys farreri* and its trans-generational immune protection to offspring against bacterial challenge. *Dev. Comp. Immunol.* 41, 569–577. doi: 10.1016/j.dci.2013.07.001

Zanchi, C., Troussard, J. P., Martinaud, G., Moreau, J., and Moret, Y. (2011). Differential expression and costs between maternally and paternally derived immune priming for offspring in an insect. *J. Anim. Ecol.* 80, 1174–1183. doi: 10.1111/j.1365-2656.2011.01872.x

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Epigenetics and Early Life Stress: Experimental Brood Size Affects DNA Methylation in Great Tits (*Parus major*)

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Early developmental conditions are known to have life-long effects on an individual's behavior, physiology and fitness. In altricial birds, a majority of these conditions, such as the number of siblings and the amount of food provisioned, are controlled by the parents. This opens up the potential for parents to adjust the behavior and physiology of their offspring according to local post-natal circumstances. However, the mechanisms underlying such intergenerational regulation remain largely unknown. A mechanism often proposed to possibly explain how parental effects mediate consistent phenotypic change is DNA methylation. To investigate whether early life effects on offspring phenotypes are mediated by DNA methylation, we cross-fostered great tit (*Parus major*) nestlings and manipulated their brood size in a natural study population. We assessed genome-wide DNA methylation levels of CpG sites in erythrocyte DNA, using Reduced Representation Bisulfite Sequencing (RRBS). By comparing DNA methylation levels between biological siblings raised in enlarged and reduced broods and between biological siblings of control broods, we assessed which CpG sites were differentially methylated due to brood size. We found 32 differentially methylated sites (DMS) between siblings from enlarged and reduced broods, a larger number than in the comparison between siblings from control broods. A considerable number of these DMS were located in or near genes involved in development, growth, metabolism, behavior and cognition. Since the biological functions of these genes line up with previously found effects of brood size and food availability, it is likely that the nestlings in the enlarged broods suffered from nutritional stress. We therefore conclude that early life stress might directly affect epigenetic regulation of genes related to early life conditions. Future studies should link such experimentally induced DNA methylation changes to expression of phenotypic traits and assess whether these effects affect parental fitness to determine if such changes are also adaptive.

Keywords: parental effects, development, postnatal conditions, growth, physiology, behavior

INTRODUCTION

Developmental phenotypic plasticity can be defined as irreversible changes in the phenotype resulting from environmentally introduced alterations in development (Forsman, 2015). These changes can occur through parental effects, which occur when the parental environment or phenotype affects that of their offspring. Parents with extended brood care are known to affect their offspring via both prenatal and postnatal effects. Well known prenatal effects are those that occurred in humans after prenatal exposure to the Dutch famine, leading to for example lower glucose tolerance (de Rooij et al., 2006), obesity (Roseboom et al., 2006), diabetes (Kahn et al., 2009) and impaired selective attention (de Rooij et al., 2010). A classic example of a postnatal parental effect is that of maternal nursing and grooming on anxiety and stress response of rat pups (Weaver et al., 2004). A likely reason why parents adjust their offspring's phenotype is to maximize parental fitness (Reddon, 2012), by transferring information about the current environment to their offspring and subsequently shape their offspring's phenotype to match the environmental conditions. If these conditions remain stable, this might increase their offspring's reproduction or survival (Champagne et al., 2003; Dantzer et al., 2013).

Parents can passively pass on information about current environmental conditions via prenatal hormone secretion (Dloniak et al., 2006; Dantzer et al., 2013) and resource allocation (de Rooij et al., 2006; Roseboom et al., 2006; Kahn et al., 2009) as has been observed in mammals or by yolk hormone deposition as observed in various bird species (Schwabl, 1993; Bentz et al., 2016). For example, yolk testosterone in wild Eastern Bluebirds (*Sialia sialis*) is positively correlated with breeding density and nestling growth (Bentz et al., 2016). Parents can also transfer information about current environmental conditions in an active way, by for example grooming behavior (Champagne et al., 2003; Weaver et al., 2004), thereby shaping early environmental conditions of the offspring after birth. Such early developmental conditions provided by the parents are known to have long-term influences on their offspring's behavior (Carere et al., 2005; van Oers et al., 2015), physiology (Keller and van Noordwijk, 1994; DeKogel, 1997; Naef-Daenzer and Keller, 1999) and may also have fitness consequences (DeKogel, 1997; Naguib et al., 2006). Intergenerational parental effects indicate an information transfer from parent to offspring (Jablonka and Raz, 2009; Bošković and Rando, 2018) but this does not imply that the patterns will be stably inherited via parental germ cells (Heard and Martienssen, 2014; Guerrero-Bosagna et al., 2018).

Epigenetic mechanisms have repeatedly been suggested to mediate the parental regulation of offspring phenotype (Kappeler and Meaney, 2010; Groothuis and Trillmich, 2011; Kilvitis et al., 2014). These biochemical mechanisms stably alter gene expression by affecting either transcription or translation without a change in the primary nucleotide sequence of the genome. Since epigenetic mechanisms can be induced in response to the local environment (Weaver et al., 2004; Pertille et al., 2017; Zimmer et al., 2017; Liu et al., 2018) they are good candidates to facilitate early developmental effects on offspring phenotype. The best-studied epigenetic mark is DNA methylation, which is the addition of a methyl group to a nucleotide. In vertebrates, this nucleotide is usually a cytosine in a CpG context, which is a CG dinucleotide (5′-cytosine guanine-3′), separated by a phosphate (p) group. Methylation can affect gene expression by interfering with the binding of proteins necessary for transcription initiation (Bird, 2002; Moore et al., 2013). Pre- and postnatal parental effects on offspring DNA methylation have been found in vertebrates like humans (Tobi et al., 2009, 2014), fish (McGhee and Bell, 2014), rats (Weaver et al., 2004) and mice (St-Cyr and McGowan, 2015), but this is hardly studied in altricial birds, even though there are some very suitable model systems.

In altricial birds in natural conditions, a major part of the early developmental conditions are largely determined by the parents, because the nestlings completely rely on their parents for nutrition. The parents are able to affect the quality and quantity of food per nestling by adjusting the egg laying date and brood size (Pettifor et al., 1988, 2001; Perrins and McCleery, 1989), prey choice, food selectivity (Wright et al., 1998; García-Navas and Sanz, 2010; Mathot et al., 2017), provisioning frequency and food allocation (Christe et al., 1996; Naef-Daenzer and Keller, 1999; Wilkin et al., 2009; Mutzel et al., 2013; van Oers et al., 2015; Caro et al., 2016). Early environmental conditions have been extensively studied in altricial birds, since offspring may experience variable natural environmental conditions that are easily manipulated experimentally, such as brood size. The effects of brood size are likely due to nutritional stress, but the direct causes of nutritional stress may depend on the provisioning tactics of the parents (Mathot et al., 2017). Parents may be unable to compensate for an increased food demand (Gow and Wiebe, 2014; Mathot et al., 2017) or may increase the feeding frequency (Hinde and Kilner, 2007; Baldan et al., 2019) but reduce prey selectivity in enlarged broods (Wright et al., 1998; García-Navas and Sanz, 2010; Mathot et al., 2017). Another cause of nutritional stress could be an increase in nestling begging costs in enlarged broods (Neuenschwander et al., 2003) due to increased social stress and competition. Brood size most prominently modifies offspring growth (Tinbergen and Boerlijst, 1990; Naguib et al., 2004; Nettle et al., 2013) and development (Naguib et al., 2004), in turn affecting fledging age (Naguib et al., 2004) and fledging size/condition (Tinbergen and Boerlijst, 1990; Sanz and Tinbergen, 1999). However, brood size has also an effect on offspring physiology, where larger brood sizes cause changes in energy metabolism (Mertens, 1969), immunocompetence (Brinkhof et al., 1999; Saino et al., 2003; Naguib et al., 2004), testosterone levels (Naguib et al., 2004), the stress response (Naguib et al., 2011) and corticosterone levels (Saino et al., 2003). Ultimately, these changes have consequences for the cognitive ability (Nettle et al., 2015) and the behavior (Carere et al., 2005; Krause et al., 2009) of offspring. However, not much is known about how these early developmental conditions shape development, physiology and behavior of the offspring in a stable manner. Changes in DNA methylation due to these early developmental conditions are a good candidate for explaining parental induced phenotypic

plasticity, but only few studies examined early environmental effects on DNA methylation in wild avian populations (Bentz et al., 2016; Rubenstein et al., 2016; Sheldon et al., 2018; Jimeno et al., 2019; Sepers et al., 2019). Only two of these studies made use of a brood size experiment. In a study on captive zebra finches (*Taeniopygia guttata*) individuals reared in large broods showed higher DNA methylation of the glucocorticoid receptor gene (Nr3c1) compared to individuals raised in small broods (Jimeno et al., 2019). Since only one candidate gene was targeted, it remains to be elucidated if more genes are affected. In another study on the same species, however, no difference in DNA methylation was detected when comparing experimentally reduced and enlarged broods using MS-AFLP (Sheldon et al., 2018). The chosen method (MS-AFLP; Reyna-López et al., 1997) has some drawbacks, it for example only screens anonymous loci and since no annotation is possible, no clear expectations can be formulated (Schrey et al., 2013). In the study of Sheldon et al. (2018), only the enlarged and reduced broods were manipulated, leaving the control broods unmanipulated. Manipulated individuals showed more hypomethylation compared to unmanipulated individuals, suggesting an effect of manipulation on DNA methylation (Sheldon et al., 2018). Thus, early developmental conditions might induce changes in nestling DNA methylation via post-natal effects such as brood size, however, to what extend early life conditions causally affect DNA methylation in functionally relevant genes is largely unknown.

Here, we experimentally manipulated brood size and assessed its effect on DNA methylation in a wild songbird species, the great tit (*Parus major*). The great tit has been a model system for ecological and evolutionary studies, with long-term studies in both wild and captive populations (Laine et al., 2016; Bosse et al., 2017; Spurgin et al., 2019). We cross-fostered 3-day old nestlings between pairs of matched broods creating enlarged broods with three nestlings extra and reduced broods with three nestlings less. In the two broods within a control pair, the original brood size of both broods remained the same, but half of the nest was cross-fostered. This classical approach has been shown to be an effective way to affect offspring behavior, physiology and body size (Sanz and Tinbergen, 1999; Neuenschwander et al., 2003; van Oers et al., 2015) and allows us to disentangle prehatching from rearing effects (Sepers et al., 2019). In birds, like most vertebrates, almost all methylation occurs at CpG sites (Derks et al., 2016; Laine et al., 2016). DNA methylation variation in the great tit has been associated with phenotypic traits such as exploratory behavior (Riyahi et al., 2015; Verhulst et al., 2016) and the onset of reproduction (Viitaniemi et al., 2019; Lindner et al., 2021a). Low levels of CpG promoter region methylation, and more specifically of sites in the transcription start site (TSS), are associated with increased gene expression in the great tit (Laine et al., 2016). We therefore used Reduced Representation Bisulfite Sequencing (RRBS) to compare CpG site-specific DNA methylation levels between siblings from enlarged and reduced broods, and between siblings from control broods. Furthermore, we used the existing annotation of the great tit reference genome to assess the functional importance of differentially methylated sites.

MATERIALS AND METHODS

Subjects, Study Site, and General Procedures

This study was conducted in April, May and June 2016 in the Boslust study population, near Arnhem, Netherlands (5°850 E, 52°010 N), a 70 ha field site consisting of mixed pine-deciduous woodlands and grassy meadows. The study site contained about 150 nest boxes, which were predominantly used by great tits. From the first week of April onward, we checked nest boxes weekly to determine initiation of breeding activity. We inspected occupied nest boxes every other day to determine the date of first egg-laying, clutch size and start of incubation, allowing us to estimate hatch dates. By visiting nests daily around the expected hatch date, we determined the exact date at which the majority of the eggs within a clutch hatched (hereafter: hatch date).

Cross-Fostering and Brood Size Manipulation

Clutches with the same hatching date (D0) and similar brood sizes were assigned to a cross-foster pair (N = 30 broods; 15 cross-foster pairs). A cross-foster pair was randomly assigned to become either a control pair or a treatment pair, independently of original brood size. When nestlings were three days old (D3), a partial cross-foster design was employed. We used the method according to van Oers et al. (2015) for cross-fostering. For this, nestlings within broods were ranked based on their weight (using a digital scale, ±0.01 g) and then randomly either the even or the odd ranked nestlings were swapped between the two broods (**Supplementary Figure 1**). In this way, differences in weight between cross-fostered nestlings and nestlings that stayed in the brood of origin were minimized (van Oers et al., 2015). For control pairs (twelve control broods; six cross-foster pairs), half of the nestlings were swapped (cross-fostered) between the two broods, while the other half stayed in the brood of origin (unmoved), without changing the original brood size. For treatment pairs (nine reduced and nine enlarged broods; nine cross-foster pairs), one brood received three nestlings more than the original brood size (+3, enlarged) and the other brood received three nestlings less than the original brood size (−3, reduced) (**Supplementary Figure 1**). We aimed for similar numbers of unmoved compared to cross-fostered nestlings in a brood and minimal weight differences between unmoved and cross-fostered siblings.

To be able to identify individuals and their brood of origin, the down tufts on the head, wings and back of the nestlings were selectively plucked right before weighing and cross-fostering (van Oers et al., 2015). This enabled us to identify the nestlings up until day six, the day at which nestlings were fitted with uniquely numbered aluminum bands (Vogeltrekstation, Netherlands).

Fourteen days after hatching (D14), a blood sample (approximately 10 μL) was taken from the brachial vein and stored in Eppendorf tubes containing one ml cell lysis buffer. The tubes were stored at the NIOO-KNAW at room temperature until further analysis. Since some broods were deserted or nestlings were missing or found dead, we were able to take blood

samples from 153 nestlings from 25 broods. Of these 25 broods nine were control broods (three complete control pairs and three single control broods) and seven were enlarged broods and nine were reduced broods (seven complete treatment pairs and two single treatment broods).

Sample Selection and Processing

From the seven treatment pairs (reduced and enlarged), matched samples from two biological siblings ($N = 14$; seven reduced and seven enlarged) were chosen for further analysis. To disentangle treatment effects on DNA methylation from biological variation in DNA methylation between the enlarged and reduced pool, we decided to compare two control pools as well. From the three control pairs, four samples ($N = 12$) per cross-foster pair were chosen for further analysis (biological sibling pairs being raised in different control broods). Since siblings are more similar to each other in their methylation profile than to nestlings from other broods (Viitaniemi et al., 2019; van Oers et al., 2020), this approach allowed us to control for prehatching differences in DNA methylation by only comparing DNA methylation levels between siblings raised in enlarged and reduced broods and between siblings raised in control broods. This approach resulted in a total sample size of 26 samples from 26 individuals (**Supplementary Table 1**).

Reduced Representation Bisulfite Sequencing Library Preparation and Sequencing

For DNA isolation, red blood cells were separated from the plasma by spinning the samples in a centrifuge at 14,000 rpm for twelve minutes Subsequently, the plasma was removed using Hamilton syringes (Merck KGaA) and the DNA was extracted from the red blood cells using FavorPrepT M 95-well Genomic DNA Kit. After DNA extraction, the DNA concentration of each individual sample was checked on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, United States). If needed, a sample was diluted to equalize the concentrations of all the samples. In addition, DNA quality was checked on agarose gels. Subsequently, the samples were pooled per treatment, resulting in a Reduced pool and an Enlarged pool (both consisting of seven individuals), and a Control1 pool and a Control2 pool (both consisting of six individuals) (**Supplementary Table 1**). The samples were pooled based on concentration, in order to minimize variation in the amount of starting DNA between individuals in one pool. Earlier research showed that pooling individuals is a reliable way to assess average group DNA methylation (Docherty et al., 2009). Within one pool all samples were, to our knowledge, unrelated. DNA isolation was finalized within one day to prevent batch effects between pools. We assessed genome-wide DNA methylation levels using Reduced Representation Bisulfite Sequencing (RRBS; Meissner, 2005). RRBS was done as mentioned in Derks et al. (2016). The genomic DNA was digested the with the enzyme Msp1. This enzyme cuts the genomic DNA at CCGG motif sites. The restriction fragments were size selected to a range of 20–200 bp, by cutting from gel after preparative gel electrophoresis. Secondly, the fragmented DNA was treated with the chemical sodium bisulfite, which turns unmethylated cytosines (C's) into uracils (U's) which will later be read as thymines (T's). Bisulfite-PCR amplification was conducted using PfuTurboCx Hotstart DNA polymerase (Stratagene) and 18 PCR cycles. The final amplified Enlarged and Reduced pools were sequenced in 2016 on an Illumina HiSeq 2500 (100 bp from single end reads), and the final amplified control pools were sequenced in 2018 on an Illumina HiSeq 4000 (100 bp from single end reads). Bisulfite sequencing was done by the Roy J. Carver Biotechnology Centre (University of Illinois at Urbana-Champaign, United States).

DNA Methylation Analysis

Quality Control and Trimming

Raw reads from the RRBS data were quality checked and checked for adapter content using FastQC v0.11.8 (Andrews, 2010). FastQ screen v0.11.1 (Wingett and Andrews, 2018) in bisulfite mode was used to detect possible contaminations with pre-existing databases and indexed genomes. The databases and genomes were Phix (*Coliphage* phi-X174), vectors (UniVec Core), *Arabidopsis thaliana* (*A. thaliana* (thale cress), TAIR10), *Escherichia coli* (*E. coli str. K-12 substr. MG1655*) and *Homo sapiens* (Genome Reference Consortium Human Build 38). After assessment of the quality checks, measures were taken to improve the quality of the reads. For this, the reads were trimmed for quality ($\geq$20 PHRED quality score), length ($\geq$20 bp for the control pools and $\geq$36 bp for the treatment pools because of high per base *N* content) and adapter sequences using Trim Galore v0.4.1 (Krueger, 2012) with the –rrbs option. The results were summarized using Multiqc 1.7 (Ewels et al., 2016). Quality improvement of the reads was verified by FastQC, FastQ screen and Multiqc. Raw reads were submitted to NCBI under the BioProject PRJNA208335 with accession numbers SRR11078101 (Enlarged pool), SRR11078100 (Reduced pool), SRR11078099 (Control1) and SRR11078098 (Control2).

Alignment and Methylation Calling

Trimmed reads were aligned to the *Parus major* reference genome v1.1[1] (Laine et al., 2016) using BS-Seeker2[2] v2.0.6 (Guo et al., 2013) with Bowtie2[3] v2.1.0 (Langmead et al., 2009) using the end-to-end alignment mode. Of the aligned reads, the methylation levels for each site were determined by dividing methylated C's of a site by the total coverage of that site (C/C + T) which was done with the methylation call script *bs_seeker2-call_methylation.py* from BS-Seeker2.

Filtering of Methylation Calls

Before the data was filtered it was transformed to fit the format of methylKit. This was done using a custom bash script and only for CpG sites. Filtering was done with R version 3.6.1 and the R package methylKit[4] v1.15.3 (Akalin et al., 2012). First, a principle component analysis (PCA) was conducted on all CpG

[1]https://www.ncbi.nlm.nih.gov/assembly/GCF_001522545.3

[2]https://github.com/BSSeeker/BSseeker2

[3]http://bowtie-bio.sf.net/bowtie2

[4]https://github.com/al2na/methylKit

sites that were present in all pools to check clustering of the four pools. The default settings of the *PCASamples* function in methylKit were used, which means that the percent methylation matrix was transposed (this is equivalent to doing PCA on variables that are sites) and that sites with low variation in DNA methylation or low coverage (<10) were discarded prior to the PCA. To test whether average CpG methylation percentage differed significantly between the four pools, a one-way analysis of variance (ANOVA) and subsequent Tukey *post hoc* analyses were conducted. Next, sites that were not present in both pools (both treatment pools or both control pools), sites with low coverage (<10) and sites with methylation levels of 0 or 100% in all pools were excluded. The treatment pools were sequenced deeper (i.e., higher coverage) than the control pools and to avoid a PCR bias in the statistical tests, we applied percentile filtering (99.9) and the coverage of the samples was normalized. After filtering of the control pools, 213,764 out of the 247,979 CpG sites that were present in both control pools could be used for further analysis. After filtering of the treatment pools, 235,618 out of the 252,698 CpG sites that were present in both treatment pools could be used for further analysis. Correlation matrixes were made to check for abnormalities.

Statistical Analysis

Differentially methylated sites (DMS) between Reduced and Enlarged and between Control1 and Control2 were also assessed using the R package methylKit v1.15.3 and R version 3.6.1 (Akalin et al., 2012). MethylKit reads the data and creates a data frame where it calculates the percentage of methylated C's at a given site from the methylation ratio created by BSSeeker2. Complementary CpG dinucleotides were not merged. Next, differential methylation per site was assessed by comparing the fraction of methylated C's between two pools using a Fisher's exact test, since there was only one pool per group for both comparisons. To minimize the chance of getting false positives, we decided to use a stringent threshold of 25% instead of 10% differential methylation. We used a Bonferroni corrected α-threshold [$-\log_{10}(0.05/213{,}764) = 6.63$ for Control1 vs. Control2 and $-\log_{10}(0.05/235{,}618) = 6.67$ for Reduced vs. Enlarged] for a site to be considered a DMS.

Gene Annotation

DMS were annotated using the *Parus major* reference genome build v1.1[5], annotation version 102[6], custom R scripts and R packages GenomicFeatures v1.30.0 (Lawrence et al., 2013) and rtracklayer v1.42.2 (Lawrence et al., 2009). Genomic regions were TSS, promoter, intron, exon, five prime untranslated region (5'UTR), three prime untranslated region (3'UTR), upstream and downstream. TSS regions were defined as 300 bp upstream to 50 bp downstream of the annotated transcription starting position of each gene (Laine et al., 2016; Viitaniemi et al., 2019). Since TSS regions overlap with promoter regions, DMS associated to a TSS region were also associated to a promoter region. In such cases, only the TSS region was reported. We defined the TSS region as in Laine et al., 2016 since in this study, low levels of CpG methylation in specifically this region were associated with increased gene expression in the great tit. Promoter regions were defined as 2000 bp upstream to 200 bp downstream of the annotated gene start site (Lindner et al., 2021a). Upstream and downstream regions were defined as 10 K bp up- and downstream regions adjacent to the gene body, respectively (Laine et al., 2016; Lindner et al., 2021b). Since DNA methylation is reciprocal on both strands, annotation was not directional (i.e., each DMS could overlap with the Watson and Crick strands). Please note that one site can be associated to multiple genes or regions, because genes can be located on opposite strands, regions within a gene can have overlapping regions depending on the transcript and genes can have opposite transcription directions. If this was the case, we checked the site in NCBI Genome Data Viewer and prioritized DMS in TSS and promoter regions, because CpG methylation within the regulatory region is known to affect gene expression in great tits (Laine et al., 2016).

Gene Ontology Analysis

The function and significance of the genes that were associated to a DMS were investigated by looking up GO terms and descriptions of the genes of chicken at uniprot[7], NCBI[8], Ensembl[9] and genecards[10]. We focused on molecular functions and biological processes. If there were no GO terms and descriptions of chicken (*Gallus gallus*) genes available, we used zebra finch (*T. guttata*) or human genes (*Homo sapiens*). Uncharacterized LOC genes were checked using NCBI and Ensembl. A LOC gene was included if its biological function could be predicted and was excluded if it was likely to be a duplication of (part of) the gene it was predicted to be, ncRNA or truly uncharacterized. We focused on DMS within regulatory regions (promoter and TSS regions) of genes and DMS that occurred in the same gene. Information about other DMS can be found in **Supplementary Tables 4–9**.

Additionally, GOrilla was used to identify enriched GO terms (Eden et al., 2009). Since there was not enough power to provide both a background and a target list, all genes in which a CpG site was found, so all genes that were covered by both pools in one comparison, were given as input. This was done for the Enlarged pool versus the Reduced pool comparison and the Control1 pool versus Control2 pool comparison separately. The genes were ranked according to how well the associated CpG site differentiated between the two pools that were compared using the *p*-value from the Fisher's exact test as described above. LOC genes were excluded by Gorilla. GOrilla was run with default running parameters (species used: *H. sapiens*; single ranked list of genes, *p*-value <0.001, GO database last updated on December 12, 2020). The FDR method was used to correct enrichment tests for multiple testing of the GO terms. GO categories were considered significantly enriched if the FDR corrected *p*-value was <0.05.

[5]https://www.ncbi.nlm.nih.gov/assembly/GCF_001522545.3

[6]https://ftp.ncbi.nlm.nih.gov/genomes/all/annotation_releases/9157/102/

[7]www.uniprot.org

[8]www.ncbi.nlm.nih.gov

[9]www.ensembl.org

[10]http://www.genecards.org

All scripts used for the bioinformatics and biostatistics steps can be found in the **Supplementary Material**.

RESULTS

General

The percentage of fully bisulfite converted reads was >99.99% in all pools (**Supplementary Table 2**). A Principle Component Analysis (PCA) revealed that 51.95% was explained by PC1, 39.07% by PC2, 8.98% by PC3 and 2.20×10^{-29}% by PC4 (**Supplementary Figure 2**). The Reduced pool and the Enlarged pool cluster together very closely along both PC1 and PC2 (**Supplementary Figure 3**). The two control pools are relatively close to one another along PC2 but vary along PC1.

Average CpG methylation percentage differed significantly between the four pools (one-way ANOVA: $F_{3,836192} = 96.8$, $p < 0.02 \times 10^{-14}$). A Tukey *post hoc* test showed that all pools differed significantly in average CpG methylation percentage: the methylation percentage was significantly lower in the Reduced pool compared to the Enlarged pool (mean ± SE; Reduced: 27.48 ± 0.0007; Enlarged: 27.76 ± 0.0007; $p = 0.04$). The methylation percentage was significantly higher in Control1 compared to Control2 (Control1: 29.13 ± 0.0008; Control2: 28.41 ± 0.0007; $p = 0.07 \times 10^{-9}$). The methylation percentages in the treatment pools were significantly lower compared to the control pools (all $p \leq 0.04 \times 10^{-7}$).

An overview of the number of reads and CpG sites before and after filtering and the mapping and calling success is given in **Supplementary Table 3**. 235,618 CpG sites were present in both treatment pools and 213,764 CpG sites were present in both control pools (after filtering). 209,049 CpG sites were shared by all four pools (**Supplementary Figure 4**).

Of the 213,764 CpG sites that were present in both control pools, 17 sites were significantly differentially methylated (**Figure 1A**), of which 14 were located on autosomes, two on the sex chromosome (chrZ) and one on a scaffold (**Figure 2A**). Of the 17 DMS, 14 sites could be annotated. Of these 14 DMS, four were located in a promoter region, of which two were located in a TSS region. Furthermore, one DMS was located in an exonic region, five in an intronic region, two in a downstream region, one in an upstream region and one DMS in both an upstream

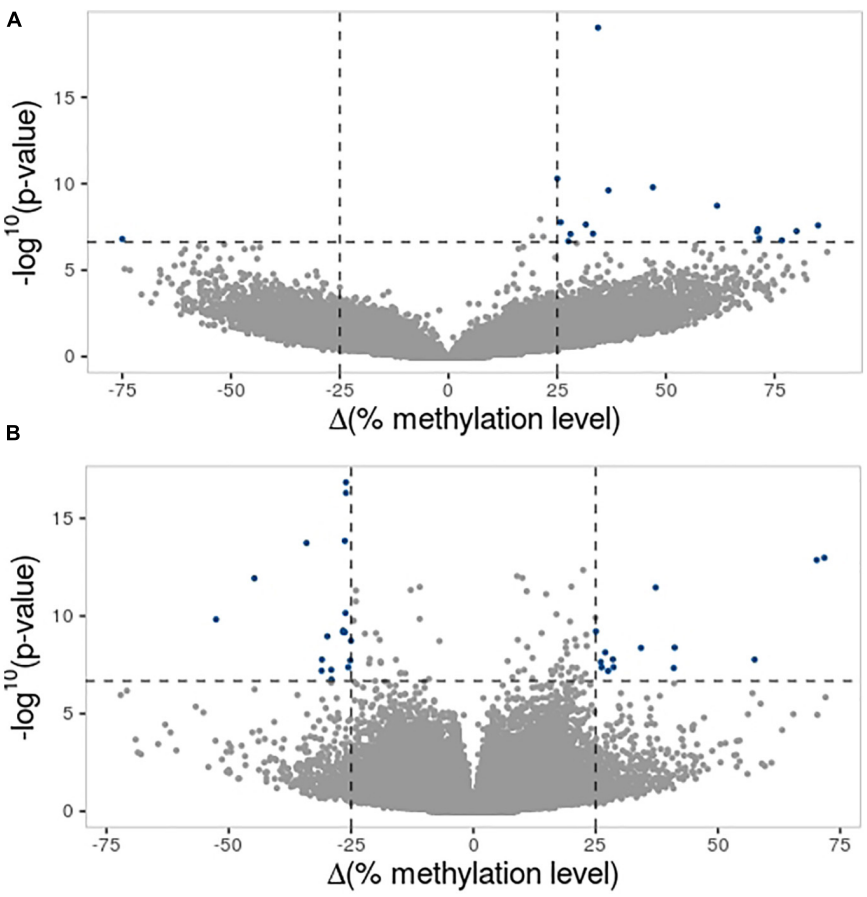

FIGURE 1 | Volcano plot of *p*-values (in $-\log_{10}$ scale) corresponding to the significance of the variation in DNA methylation between two pools. Each dot represents a CpG site tested for differential methylation. Dark blue dots represent CpG sites that differ significantly between the pools with a minimal difference of 25% differential methylation. Dotted horizontal lines marks the genome wide significance threshold. Dotted vertical lines represent 25% differential methylation. **(A)** Control1 versus Control2, (*n* = 213,764). **(B)** Reduced versus Enlarged, (*n* = 235,618).

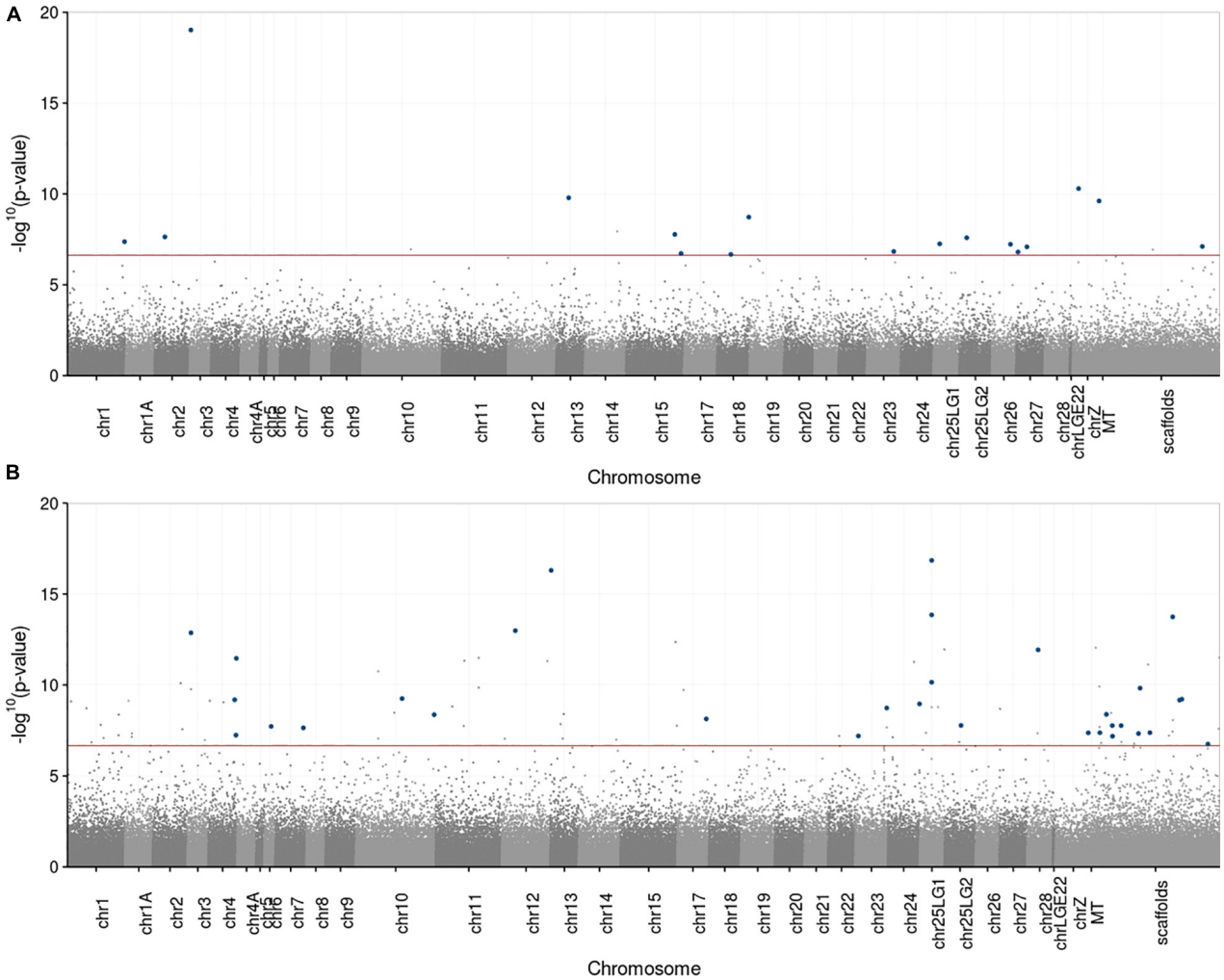

FIGURE 2 | Manhattan plot of *p*-values (in $-\log_{10}$ scale) corresponding to the significance of the variation in DNA methylation between the pools. Each dot represents a CpG site tested for differential methylation. Dark blue dots represent CpG sites that differ significantly between the pools with a minimal difference of 25% differential methylation. The dotted red line marks the genome wide significance threshold. The sites are plotted against the location of the associated site within the genome. Alternating colors help to differentiate adjacently displayed chromosomes. ChrZ is a sex chromosome, all the other chromosomes are autosomes. All unplaced scaffolds are merged into the category "scaffolds". **(A)** Control1 versus Control2, (*n* = 213,764). **(B)** Reduced versus Enlarged, (*n* = 235,618).

and downstream region (**Supplementary Table 4**). The 14 DMS were located in or nearby 15 genes (**Supplementary Table 5**).

Of the 235,618 CpG sites that were present in both treatment pools, 32 sites were significantly differentially methylated (**Figure 1B**), of which 20 were located on autosomes, one on the sex chromosome and 12 on scaffolds (**Figure 2B**). Of the 32 DMS, 23 could be annotated. Of these 23 DMS, nine were located in a promoter region, of which three in a TSS region. Furthermore, three DMS were located in an exonic regions, eight DMS were located in an intronic region, two in a downstream region and one in an upstream region (**Supplementary Table 4**). The 23 DMS were located in or nearby 21 genes (**Supplementary Table 6**).

None of the 17 DMS between Control1 pool and Control2 pool were significantly differentially methylated between the Reduced pool and the Enlarged pool (**Supplementary Figure 5** and **Supplementary Table 7**). Out of the 17 DMS, 11 were covered in the Reduced pool and the Enlarged pool. None of the 32 DMS between the Reduced pool and the Enlarged pool were significantly differentially methylated between Control1 pool and Control2 pool (**Supplementary Figure 5** and **Supplementary Table 7**). Out of the 32 DMS, 26 were covered in both Control pools.

Differentially Methylated Sites in Genes and in Regulatory Regions

Control Pools

When comparing the two control pools, none of the DMS occurred within the same genes. All of these DMS were hypermethylated, which translates to a higher methylation percentage in Control1 compared to Control2. We found four

TABLE 1 | Full name, gene name, location, chromosome number or unplaced scaffold, Δ of % methylation level, *p*-value, region and biological functions associated with DMS between Control1 pool and Control2 pool.

Location DMS (chrom./scaf.)	Δ meth. (*p*-value)	Region	Biological functions
Heat shock protein family A (Hsp70) member 2 (*HSPA2*)			
54944183 (chr5)	26% (1.68×10^{-8})	Promoter	Protein refolding, spermatogenesis
Methyltransferase like 8 (*METTL8*)			
17727814 (chr7)	28% (2.12×10^{-7})	Promoter	Skeletal muscle tissue development, fat cell differentiation, histone acetylation, mRNA methylation
Zinc finger SWIM domain-containing protein 1 (*ZSWIM1*-like; *LOC107213098*)			
6759952 (chr20)	28% (8.15×10^{-8})	TSS	Zinc and metal ion binding
Oncostatin-M-specific receptor subunit beta-like (*OSMR*-like; *LOC107198170*)			
11977179 (chrZ)	25% (5.05×10^{-11})	TSS	Cell population proliferation, inflammatory response

DMS to be situated in regulatory regions (**Table 1**). These DMS were located in the promoter region of heat shock protein family A member *2* (*HSPA2*), in the promoter region of methyltransferase like 8 (*METTL8*), around the TSS region of the predicted gene zinc finger SWIM domain-containing protein 1 (*ZSWIM1*-like; *LOC107213098*) and around the TSS region of the predicted gene oncostatin-M-specific receptor subunit beta-like (*OSMR*-like; *LOC107198170*).

Out of all 15,603 covered genes, 11,182 were recognized by GOrilla. We detected 98 enriched GO terms for the ontology biological process, 28 for the molecular functions and 16 for cellular component (*p*-value <0.001). After FDR correction, 47 of the biological function GO terms were significantly enriched, 19 of the molecular function and eight of the cellular component (FDR *q*-value <0.05) (**Supplementary Table 10**). The most significant GO terms were anatomical structure development (GO:0048856, FDR = 1.95×10^{-8}), developmental process (GO:0032502, FDR = 7.28×10^{-6}) and system development (GO:0048731, FDR = 5.09×10^{-5}). Of all genes (excluding the LOC-genes) in which a DMS was found, only *HSPA2* was associated with the significantly enriched GO terms.

Reduced Versus Enlarged

In the treatment comparison, three DMS were found within the same gene (**Table 2**). These three DMS were hypomethylated, which translated to a lower methylation percentage in the Enlarged pool than in the Reduced pool. Of these three DMS, one was situated in the exonic region and two in the intronic region of laminin subunit gamma 3 (*LAMC3*). Nine DMS were situated in the regulatory regions of genes and none of these DMS occurred in the same gene (**Table 2**). Of these nine DMS, five were hypermethylated, which translated to a higher methylation percentage in the Enlarged pool than in the Reduced pool. These DMS were situated around the TSS region of tissue specific transplantation antigen P35B (*TSTA3*), around the TSS region of the predicted gene ketosamine-3-kinase-like (*FN3KRP*-like; *LOC107198385*), in the promoter region of prominin *2* (*PROM2*), around the TSS region of zinc finger protein 664-like (*ZNF664*-like; *LOC107199222*) and in the promoter region of plectin-like (*PLEC*-like, *LOC107199333*). The four remaining DMS in regulatory regions of genes were hypomethylated, which translated to a lower methylation percentage in the Enlarged pool than in the Reduced pool. These DMS were situated in the promoter region of complement C1q subcomponent subunit C-like (*C1QC*-like; *LOC107213704*), in the promoter region of the gene activating transcription factor 6 (*ATF6*), in the promoter region of prolactin regulatory element binding (*PREB*) and in the promoter region of WD repeat domain 83 opposite strand (*WDR83OS*).

Out of all 15,718 covered genes, 11,210 were recognized by GOrilla. We detected 82 enriched GO terms for the ontology biological process, 23 for the molecular functions and seven for cellular component (*p*-value <0.001). After FDR correction, 42 of the biological function GO terms were significantly enriched, eleven of the molecular function and one of the cellular component (FDR *q*-value <0.05) (**Supplementary Table 11**). The most significant GO terms were developmental process (GO:0032502, FDR = 1.64×10^{-5}), regulation of multicellular organismal process (GO:0051239, FDR = 6.72×10^{-5}), anatomical structure morphogenesis (GO:0009653, FDR = $4{,}71 \times 10^{-5}$), anatomical structure development (GO:0048856, FRD = 1.09×10^{-4}) and regulation of cell differentiation (GO:0045595, FDR = 9.59×10^{-5}). All genes (excluding the LOC-genes) in which a DMS was found were associated with the significantly enriched GO terms.

The results that were obtained with a less stringent threshold of 10% differential methylation are reported in **Supplementary Tables 12–16**.

DISCUSSION

The mechanisms underlying intergenerational regulation of developmental phenotypic plasticity in birds remain largely unknown, but recent studies indicate a role for DNA methylation (Bentz et al., 2016; Sheldon et al., 2018). Here, we explored this further by experimentally manipulating brood size in a partial cross-foster experiment and assessing the effect of experimental brood size on DNA methylation in a wild songbird species, the great tit. We found more CpG sites in red blood cells to be differentially methylated between biological sibling-pairs raised in experimentally enlarged and reduced broods, than between siblings raised in partially cross-fostered control broods with unchanged brood size. Since differential DNA methylation is more apparent between nestlings from enlarged and reduced broods than between nestlings from control broods,

TABLE 2 | Full name, gene name, location, chromosome number or unplaced scaffold, Δ of % methylation level, *p*-value, region and biological functions associated with DMS between the Reduced pool and the Enlarged pool.

Location DMS (chrom./scaf.)	Δ meth. (*p*-value)	Region	Biological functions
Laminin subunit gamma 3 (*LAMC3*)			
4729995 (chr17)	-26 (1.42×10^{-14})	Intron	Cell morphogenesis, cell differentiation, visual perception, astrocyte development, retina development in camera-type eye
4730000 (chr17)	-26 (7.06×10^{-11})	Intron	
4730148 (chr17)	-26 (1.42×10^{-17})	Exon	
Tissue specific transplantation antigen P35B (*TSTA3*)			
149822731 (chr2)	34 (4.28×10^{-9})	TSS	T cell mediated cytotoxicity, nucleotide-sugar biosynthetic process
Complement C1q subcomponent subunit C-like (*C1QC*-like; *LOC107213704*)			
1797839 (chr21)	-45 (1.17×10^{-12})	Promoter	Negative regulation granulocyte differentiation, negative regulation macrophage differentiation, synapse pruning
Prominin 2 (*PROM2*)			
730012 (chr22)	70 (1.36×10^{-13})	Promoter	Cell projection organization, protein phosphorylation, regulation of GTPase activity (signal transduction)
Ketosamine-3-kinase-like (*FN3KRP*-like; *LOC107198385*)			
70917608 (chrZ)	26 (4.27×10^{-8})	TSS	Post-translational protein modification (phosphorylation)
Activating transcription factor 6 (*ATF6*)			
246819 (scaffold)	-29 (1.77×10^{-7})	Promoter	Eye development, regulation transcription, unfolded protein response (endoplasmic reticulum stress), cell apoptosis
Prolactin regulatory element binding (*PREB*)			
50860 (scaffold)	-34 (1.82×10^{-14})	Promoter	Protein exit from endoplasmic reticulum
Zinc finger protein 664-like (*ZNF664*-like; *LOC107199222*)			
19200 (scaffold)	29 (4.18×10^{-8})	TSS	–
WD repeat domain 83 opposite strand (*WDR83OS*)			
11884 (scaffold)	-53 (1.49×10^{-10})	Promoter	Phosphorylation, MAPK cascade, (m)RNA splicing, mRNA processing
Plectin-like (*PLEC*-like; *LOC107199333*)			
1772 (scaffold)	28 (6.52×10^{-8})	Promoter	–

–, The biological function could not be predicted.

this indicates that experimental variation in brood size affects DNA methylation. Furthermore, we found for the enlarged versus reduced comparison more differentially methylated CpG sites to be situated in regulatory regions (promoter and TSS regions) than for the control brood comparison. Since CpG methylation within the regulatory region is known to affect gene expression in great tits (Laine et al., 2016), we expect more functional differences in gene expression between nestlings from enlarged and reduced broods than between nestlings from control broods.

The average CpG methylation percentage differed between pools of nestlings from the enlarged and the reduced broods and between the control broods. Nestlings from experimentally enlarged and reduced broods were hypomethylated compared to nestlings from control broods. This indicates that any manipulation of brood size affected CpG methylation. A similar result was found in the study of Sheldon et al. (2018). Here, zebra finch nestlings from reduced and enlarged broods showed more hypomethylation compared to control nestlings. However, in this study, the control broods were completely unmanipulated, whereas in our study nestlings from control broods were also cross-fostered. Moreover, we found that nestlings from experimentally enlarged broods were hypermethylated compared to nestlings from reduced broods. This result supports the hypermethylation of Nr3c1 in zebra finches reared in large broods (Jimeno et al., 2019) and the positive correlation between natal brood size and the percentage of DNA methylation in Sheldon et al. (2018). However, this result does not match the lack of difference in methylation between nestlings from experimentally reduced and enlarged broods (Sheldon et al., 2018). This might be caused by the targeted approach we used in this study, compared to the MS-AFLP approach. In spite of our targeted approach, slight differences in methylation remain undetected when average DNA methylation levels are compared. Since DNA methylation is very gene- and region specific, it is important to assess site specific differences in possibly functionally relevant genes as well. Furthermore, the difference in methylation percentage between nestlings from reduced and enlarged broods was only 0.28% and the difference between nestlings that experienced a manipulation in brood size and nestlings from control broods ranged from 0.65 to 1.65%, also minimal differences. It has to be elucidated if such small differences are large enough to result in differential gene expression. Hence, it is unknown if such small differences hold any biological significance or are just caused by a statistical artifact.

As mentioned above, the biological functions of the genes and the possible consequences of methylation for gene expression will be discussed below. Since low levels of CpG promoter region methylation, and more specifically of sites in the TSS region, are associated with increased gene expression in the great tit (Laine et al., 2016), hypermethylated DMS are expected to be

associated with lower gene expression and hypomethylated DMS are expected to be associated with higher gene expression. The limitations of such a generalization will also be discussed.

Sites that were differentially methylated in the enlarged versus reduced comparison, were mainly found to be related to development, metabolism and behavior and cognition. This involved sites in the genes *LAMC3*, *PREB*, *PROM2*, *TSTA3*, *ATF6*, *FN3KRP*-like and *WDR83OS*. The biological functions of *ZNF664*-like and *PLEC*-like could not be predicted because these LOC genes were likely to be a duplication of (part of) the genes they were predicted to be or ncRNA. In the gene *LAMC3*, three different DMS were found, although none of them occurred in the regulatory region of the gene, indicating that we have no proof for a possible change in gene expression in the great tit (Laine et al., 2016). The DNA methylation levels in all three sites were higher in the reduced pool in comparison with the enlarged pool, cautiously indicating lower expression of *LAMC3* in nestlings from reduced broods. Since *LAMC3* expression is relatively high during human development (Barak et al., 2011) and low to moderate in adulthood (Hawrylycz et al., 2012; Zeng et al., 2012), this might mean that the nestlings from the reduced broods were further developed than the nestlings from the enlarged broods, which is in the expected direction based on previously found effects of brood size and food availability on development (Nettle et al., 2013) and condition (Tinbergen and Boerlijst, 1990; DeKogel, 1997; Sanz and Tinbergen, 1999). In addition, we found one DMS in the genes *PREB*, *PROM2*, *TSTA3*, *ATF6*, *FN3KRP*-like and *WDR83OS*. The DMS in both *PROM2* and *TSTA3* were hypermethylated in the enlarged pool compared to the reduced pool, suggesting lower gene expression in nestling from the enlarged broods. In the case of *PROM2*, this might indicate lower levels of cholesterol (Singh et al., 2013) in the nestlings from enlarged broods. In the case of *TSTA3*, this might indicate a lower growth potential (Willson et al., 2018) of nestlings in the enlarged broods and an effect on (bone) metabolism (Johnsson et al., 2015). The DMS in *PREB* was hypomethylated in the enlarged group, suggesting higher expression of these genes in nestlings from the enlarged group. Higher *PREB* expression might lead to lower prolactin (PRL) expression (Hiyama et al., 2015), which might indicate later sexual maturity and lower body weights (Bhattacharya et al., 2011) in nestlings from the enlarged broods. Overall, the DMS in *PREB*, *PROM2* and *TSTA3* indicate that the nestlings from the enlarged broods weighed less, developed slower and adjusted their metabolism. These effects are in the expected direction based on previously found effects of brood size on nestling condition (DeKogel, 1997; Sanz and Tinbergen, 1999), weight (Tinbergen and Boerlijst, 1990; DeKogel, 1997) and resting metabolic rate (Verhulst et al., 2006).

The genes *ATF6*, *PREB*, *FN3KRP*-like and *WDR83OS* suggest an effect of brood size on insulin-glucose metabolism specifically. The DMS in *PREB*, *ATF6* and *WDR83OS* were hypomethylated in the enlarged pool compared to the reduced pool, suggesting higher gene expression in nestlings from the enlarged broods. This indicates in the case of *ATF6* glucose intolerance (Barbosa et al., 2016), in the case of *WDR83OS* increased levels of insulin (Kesherwani et al., 2017) and in the case of *PREB* higher insulin sensitivity (Park et al., 2018) in nestlings from the enlarged broods. The function of *FN3KRP*-like function is not fully understood (Szwergold et al., 2011), although the hypermethylated DMS in the enlarged pool suggests lower gene expression in nestlings from the enlarged broods, which indicates differences in glucose metabolism (Sajuthi et al., 2016) between nestlings from the reduced and enlarged broods. The effects are in the expected direction based on previously found effects of brood size on energy metabolism (Mertens, 1969) and the results indicate food scarcity in the enlarged broods. The specific effect of food scarcity on insulin-glucose metabolism has to be elucidated yet, because the effect is dependent on the developmental stage of an individual (Gardner et al., 2005; Tobi et al., 2009). Nevertheless, insulin-glucose metabolism might be a way of dealing with nutritional constraint in the enlarged broods, allowing for growth under poor food conditions (Gardner et al., 2004, 2005). Similar results have been found before. For example, women exposed to the Dutch famine during gestation gave birth to individuals with lower glucose tolerance during adulthood, probably caused by impaired insulin secretion (de Rooij et al., 2006). This is thought to be the result of fetal adaptations to scarcity i.e., the thrifty phenotype (Hales and Barker, 1992), which becomes maladaptive when an individual is exposed to an abundance of food later in life (Stanner and Yudkin, 2001; Schulz, 2010). In humans prenatally exposed to famine, but exposed to an abundance of food later in life, this has led to higher rates of obesity (Roseboom et al., 2006) and diabetes (Kahn et al., 2009). Furthermore, differentially methylated regions were found in whole blood when compared to their siblings and these regions were associated to prenatal malnutrition, early development, metabolism and growth (Tobi et al., 2009, 2014). Thus, the consequences of early life conditions might be mediated by adjusting metabolic efficiency and DNA methylation might be one of the mechanisms behind this.

One gene, *C1QC*, is a regulator of the immune response and synapse development and was previously associated to cognition and behavior. *C1QC* expression has been associated to Alzheimer's disease and alterations in learning behavior (Khoonsari et al., 2016; Haure-Mirande et al., 2019), ADHD and autism (Corbett et al., 2007; Trent et al., 2014). However, the direction of the effect is not completely understood and might be dependent on the developmental stage (Davies et al., 2009; Trent et al., 2014). The DMS in *C1QC*-like was hypermethylated in the reduced pool compared to the enlarged pool, suggesting lower *C1QC*-like expression in nestlings from the reduced broods. Although the role of *C1QC* in development of Alzheimer's disease, ADHD and autism is not fully understood yet, this might indicate a difference in brain development, synaptic structure, behavior and cognition between the two treatment groups. This is expected based on previously found effects of food availability, diet quality and brood size on behavior (Carere et al., 2005; Krause et al., 2009; van Oers et al., 2015) and cognition (Nettle et al., 2015) in birds. Food availability is known to affect nestling stress response (van Oers et al., 2015) and exploratory behavior (Carere et al., 2005) and diet quality affects the latency to approach food and feed later in life (Krause et al., 2009). Furthermore, small natal brood size has been associated

to slow conditioning to a stimulus and slow reversal learning (Nettle et al., 2015).

The genes and their functions described above are supported by the significantly enriched GO terms developmental process, regulation of multicellular organismal process, anatomical structure morphogenesis, anatomical structure development and regulation of cell differentiation, which all indicate a difference in development between nestlings from enlarged and reduced broods. Although more GO terms were significantly enriched in the comparison of the two control pools and these were similar to those in the comparison of the two treatment pools, more GO terms in the comparison of the treatment pools were highly significantly enriched. This means that more GO terms were highly enriched when comparing reduced with enlarged nestlings than when comparing nestlings from control broods.

Overall, we show that experimental brood size variation leads to more differential DNA methylation in more regulatory regions of genes than when performing a control experiment. This indicates that DNA methylation in response to experimental variation in brood size has the potential to alter gene expression. Most of the genes were functional in tissues other than blood and this may affect how gene expression is related to a trait, due to tissue differentiation. However, multiple studies have shown that gene expression levels in the blood were related to the processes associated with that gene (Roulin et al., 2011; Zhu et al., 2017; Lindner et al., 2021b), suggesting that gene expression levels in blood in our study could explain plasticity in phenotypic traits related to brood size variation. Although these results demonstrate that early life stress affects epigenetic regulation of genes related to brood size, namely genes that are known to affect development, growth, metabolism, behavior and cognition, future work is needed. Further work should assess the causal effects of changes in DNA methylation on gene expression at these loci and related phenotypic traits, and specifically to find repeatable results in similar experiments. Furthermore, it should be assessed whether a single DMS can affect gene expression.

The expectation was that we would not find any differentially methylated sites when comparing pools of control siblings raised in different broods. Still, we found several DMS in the regulatory regions of the genes *HSPA2*, *METTL8*, *ZSWIM1*-like (*LOC107213098*) and *OSMR*-like (*LOC107198170*). These results show that our control pools were not identical in terms of DNA methylation or that these results are false positives. However, we expect the chance of these DMS to be false positives to be very slim because of our stringent approach during data analysis; we applied coverage filtering, a threshold of 25% differential methylation and a Bonferroni corrected α-threshold. Furthermore, we cannot link these differences to our experimental approach. A previous study suggested an effect of manipulation (i.e., being moved to a different nest) on DNA methylation in zebra finches (Sheldon et al., 2018), which was controlled for in the current experimental approach. This means that this cannot explain the DMS and the direction of methylation in the control comparison. One likely explanation may be the existence of large individual variation in DNA methylation (Viitaniemi et al., 2019). By pooling individuals we tried to focus on average group DNA methylation (Docherty et al., 2009) instead of individual variation. However, the number of individuals in our pools might have been too small to completely discard such individual differences. This suggests that some of our DMS in the treatment comparisons might have also been caused by individual differences. Nevertheless, PCA revealed that the reduced pool and the enlarged pool clustered together very closely, which indicates that our experimental setup, matching sibling pairs that were raised in differently sized broods, worked, since these pools were very comparable.

The fact that our control pools were not as similar as thought was supported by the finding that the hyper/hypo distribution between the control broods is non-equally divided with all differentially methylated sites being hypermethylated in Control1. This indicates that by chance some factor that induces DNA methylation in a certain direction was present in one pool. Furthermore, the two control pools did not perfectly cluster together in the PCA plot, unlike the reduced and enlarged pools. This is surprising, since we would expect control pools to cluster in the same way, since siblings were paired there as well. We therefore conclude that the controls pools were not balanced, causing the number of DMS to be higher than if the control pools would have been balanced. One reason could be a biased sex ratio (Natt et al., 2014). However, only one DMS in a regulatory region was situated on the sex chromosome, which was equal to the number found in the treatment comparison. One DMS was found in *HSPA2*, a gene involved in protein refolding and in spermatogenesis. Given the importance of this gene for male fertility (Dix et al., 1996; Son et al., 1999), this might indicate a difference in expression between the pools, which might be caused by a biased sex ratio in the individuals included in the pools, although this methylation change does not have to be sex dependent. In great tits, visual determination of the sex before the first molt is unreliable, and most birds from this study were not recaptured after the first molt and have therefore not been molecularly sexed, making it impossible to balance the number of males and females in the pools. Since a DMS in *HSPA2* might also indicate a bias in brood temperature, because *HSPA2* expression is affected by temperature in chicken testes (Wang et al., 2013, 2015), we do not expect our main findings to be affected by a potential sex-bias. Another possible, but unlikely explanation could be that the differences were caused by genetic variation between the pools, since a large fraction of erythrocyte DNA methylation is similar between relatives (Viitaniemi et al., 2019; van Oers et al., 2020). However, since the samples in one pool were, to our knowledge, unrelated and the samples in Control1 originated from siblings of the samples in Control2, we expect the genetic diversity to be larger within than between the pools, although we did not check for extra-pair paternity, which is estimated at about 10% for this population (van Oers et al., 2008). Therefore, it could be that nestlings of one sibling pair were half-siblings, because they were sired by different males. However, since we do not have individual DNA methylation information, we can only speculate on these causes and functional validation of the candidate loci is needed to assess the causal relationship between our experiment and the change in methylation, highlighting the need for studies that assess individual variation in DNA methylation levels.

Although, with our design, we cannot disentangle these possible alternative explanations explaining the high number of DMS and the direction of methylation in the control comparison. However, since the two treatment pools were, unlike the two control pools, very similar, we do think that the difference between the number of DMS in the control comparison and the treatment comparison is conservative rather than exaggerated. Therefore, we conclude that most of the DMS in the treatment comparison are likely related to the treatment and the DMS between the control pools may also be caused by some non-explained bias in the control pools.

In conclusion, to our knowledge, this is the first study that investigates the effects of experimentally altered brood size on genome-wide DNA methylation in a wild bird population and that disentangles prehatching from rearing effects with a partial cross-foster design, controls for possible effects of manipulation and assesses the functionality of annotated differentially methylated sites. Our work demonstrates that early life stress due to variation in brood size directly affects epigenetic regulation of genes that are known to affect brood-size dependent phenotypes, such as development, growth, metabolism, behavior and cognition. Although future studies are needed to validate our findings, this study underlines the potential role for DNA methylation in the intergenerational regulation of developmental phenotypic plasticity in altricial birds.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/biosample/14093678; https://www.ncbi.nlm.nih.gov/biosample/14093679; https://www.ncbi.nlm.nih.gov/biosample/14093680; and https://www.ncbi.nlm.nih.gov/biosample/14093681.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (NIOO-IvD) and the Koninklijke Nederlandse Akademie van Wetenschappen – Dier Experimenten Commissie (KNAW-DEC licenses NIOO 10.07 and 14.11 to KO).

AUTHOR CONTRIBUTIONS

BS and KO designed the study and obtained funding. BS conducted the experiment. BS and JE conducted the data analysis with help of FG and VL. BS, JE, and KO drafted the manuscript. KO supervised the study. All authors contributed to editing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.609061/full#supplementary-material

REFERENCES

Akalin, A., Kormaksson, M., Li, S., Garrett-Bakelman, F. E., Figueroa, M. E., Melnick, A., et al. (2012). methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biol.* 13:9. doi: 10.1186/gb-2012-13-10-R87

Andrews, S. (2010). *FastQC: A Quality Control Tool for High Throughput Sequence Data*. Available http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed October 2, 2019).

Baldan, D., Curk, T., Hinde, C. A., and Lessells, C. M. (2019). Alternation of nest visits varies with experimentally manipulated workload in brood-provisioning great tits. *Anim. Behav.* 156, 139–146. doi: 10.1016/j.anbehav.2019.08.004

Barak, T., Kwan, K. Y., Louvi, A., Demirbilek, V., Saygi, S., Tuysuz, B., et al. (2011). Recessive LAMC3 mutations cause malformations of occipital cortical development. *Nat. Genet.* 43, 590–U132. doi: 10.1038/ng.836

Barbosa, T. D., Ingerslev, L. R., Alm, P. S., Versteyhe, S., Massart, J., Rasmussen, M., et al. (2016). High-fat diet reprograms the epigenome of rat spermatozoa and transgenerationally affects metabolism of the offspring. *Mol. Metab.* 5, 184–197. doi: 10.1016/j.molmet.2015.12.002

Bentz, A. B., Sirman, A. E., Wada, H., Navara, K. J., and Hood, W. R. (2016). Relationship between maternal environment and DNA methylation patterns of estrogen receptor alpha in wild Eastern Bluebird (*Sialia sialis*) nestlings: a pilot study. *Ecol. Evol.* 6, 4741–4752. doi: 10.1002/ece3.2162

Bhattacharya, T. K., Chatterjee, R. N., Sharma, R. P., Niranjan, M., Rajkumar, U., and Reddy, B. L. N. (2011). Polymorphism in the prolactin promoter and its association with growth traits in chickens. *Biochem. Genet.* 49, 385–394. doi: 10.1007/s10528-010-9415-3

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes Dev.* 16, 6–21. doi: 10.1101/gad.947102

Bošković, A., and Rando, O. J. (2018). Transgenerational epigenetic inheritance. *Annu. Rev. Genet.* 52, 21–41. doi: 10.1146/annurev-genet-120417-031404

Bosse, M., Spurgin, L. G., Laine, V. N., Cole, E. F., Firth, J. A., Gienapp, P., et al. (2017). Recent natural selection causes adaptive evolution of an avian polygenic trait. *Science* 358, 365–368. doi: 10.1126/science.aal3298

Brinkhof, M. W. G., Heeb, P., Kölliker, M., and Richner, H. (1999). Immunocompetence of nestling great tits in relation to rearing environment and parentage. *Proc. R. Soc. Lond. B Biol. Sci.* 266, 2315–2322. doi: 10.1098/rspb.1999.0925

Carere, C., Drent, P. J., Koolhaas, J. M., and Groothuis, T. G. G. (2005). Epigenetic effects on personality traits: early food provisioning and sibling competition. *Behaviour* 142, 1329–1355.

Caro, S. M., Griffin, A. S., Hinde, C. A., and West, S. A. (2016). Unpredictable environments lead to the evolution of parental neglect in birds. *Nat. Commun.* 7:10. doi: 10.1038/ncomms10985

Champagne, F. A., Francis, D. D., Mar, A., and Meaney, M. J. (2003). Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiol. Behav.* 79, 359–371. doi: 10.1016/S0031-9384(03)00149-5

Christe, P., Richner, H., and Oppliger, A. (1996). Begging, food provisioning, and nestling competition in great tit broods infested with ectoparasites. *Behav. Ecol.* 7, 127–131. doi: 10.1093/beheco/7.2.127

Corbett, B. A., Kantor, A. B., Schulman, H., Walker, W. L., Lit, L., Ashwood, P., et al. (2007). A proteomic study of serum from children with autism showing differential expression of apolipoproteins and complement proteins. *Mol. Psychiatry* 12, 292–306. doi: 10.1038/sj.mp.4001943

Dantzer, B., Newman, A. E. M., Boonstra, R., Palme, R., Boutin, S., Humphries, M. M., et al. (2013). Density triggers maternal hormones that increase adaptive offspring growth in a wild mammal. *Science* 340, 1215–1217. doi: 10.1126/science.1235765

Davies, W., Humby, T., Kong, W., Otter, T., Burgoyne, P. S., and Wilkinson, L. S. (2009). Converging pharmacological and genetic evidence indicates a role for steroid sulfatase in attention. *Biol. Psychiatry* 66, 360–367. doi: 10.1016/j.biopsych.2009.01.001

de Rooij, S. R., Painter, R. C., Phillips, D. I. W., Osmond, C., Michels, R. P. J., Godsland, I. F., et al. (2006). Impaired insulin secretion after prenatal exposure to the dutch famine. *Diabetes Care* 29, 1897–1901. doi: 10.2337/dc06-0460

de Rooij, S. R., Wouters, H., Yonker, J. E., Painter, R. C., and Roseboom, T. J. (2010). Prenatal undernutrition and cognitive function in late adulthood. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16881–16886. doi: 10.1073/pnas.1009459107

DeKogel, C. H. (1997). Long-term effects of brood size manipulation on morphological development and sex-specific mortality of offspring. *J. Anim. Ecol.* 66, 167–178.

Derks, M. F. L., Schachtschneider, K. M., Madsen, O., Schijlen, E., Verhoeven, K. J. F., and van Oers, K. (2016). Gene and transposable element methylation in great tit (*Parus major*) brain and blood. *BMC Genomics* 17:13. doi: 10.1186/s12864-016-2653-y

Dix, D. J., Allen, J. W., Collins, B. W., Mori, C., Nakamura, N., PoormanAllen, P., et al. (1996). Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3264–3268. doi: 10.1073/pnas.93.8.3264

Dloniak, S. M., French, J. A., and Holekamp, K. E. (2006). Rank-related maternal effects of androgens on behaviour in wild spotted hyaenas. *Nature* 440, 1190–1193. doi: 10.1038/nature04540

Docherty, S. J., Davis, O. S. P., Haworth, C. M. A., Plomin, R., and Mill, J. (2009). Bisulfite-based epityping on pooled genomic DNA provides an accurate estimate of average group DNA methylation. *Epigenet. Chromatin* 2:3. doi: 10.1186/1756-8935-2-3

Eden, E., Navon, R., Steinfeld, I., Lipson, D., and Yakhini, Z. (2009). GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics* 10:48. doi: 10.1186/1471-2105-10-48

Ewels, P., Magnusson, M., Lundin, S., and Käller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* 32, 3047–3048. doi: 10.1093/bioinformatics/btw354

Forsman, A. (2015). Rethinking phenotypic plasticity and its consequences for individuals, populations and species. *Heredity* 115, 276–284. doi: 10.1038/hdy.2014.92

García-Navas, V., and Sanz, J. J. (2010). Flexibility in the foraging behavior of blue tits in response to short-term manipulations of brood size. *Ethology* 116, 744–754. doi: 10.1111/j.1439-0310.2010.01788.x

Gardner, D. S., Pearce, S., Dandrea, J., Walker, R., Ramsay, M. M., Stephenson, T., et al. (2004). Peri-implantation undernutrition programs blunted angiotensin ii evoked baroreflex responses in young adult sheep. *Hypertension* 43, 1290–1296. doi: 10.1161/01.HYP.0000126991.67203.7b

Gardner, D. S., Tingey, K., Van Bon, B. W. M., Ozanne, S. E., Wilson, V., Dandrea, J., et al. (2005). Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 289, R947–R954. doi: 10.1152/ajpregu.00120.2005

Gow, E. A., and Wiebe, K. L. (2014). Responses by central-place foragers to manipulations of brood size: parent flickers respond to proximate cues but do not increase work rate. *Ethology* 120, 881–892. doi: 10.1111/eth.12259

Groothuis, T. G. G., and Trillmich, F. (2011). Unfolding personalities: the importance of studying ontogeny. *Dev. Psychobiol.* 53, 641–655. doi: 10.1002/dev.20574

Guerrero-Bosagna, C., Morisson, M., Liaubet, L., Rodenburg, T. B., de Haas, E. N., Kostal, L., et al. (2018). Transgenerational epigenetic inheritance in birds. *Env. Epigenet.* 4:dvy008. doi: 10.1093/eep/dvy008

Guo, W. L., Fiziev, P., Yan, W. H., Cokus, S., Sun, X. G., Zhang, M. Q., et al. (2013). BS-Seeker2: a versatile aligning pipeline for bisulfite sequencing data. *BMC Genomics* 14:8. doi: 10.1186/1471-2164-14-774

Hales, C. N., and Barker, D. J. P. (1992). Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* 35, 595–601. doi: 10.1007/BF00400248

Haure-Mirande, J.-V., Wang, M., Audrain, M., Fanutza, T., Kim, S. H., Heja, S., et al. (2019). Integrative approach to sporadic Alzheimer's disease: deficiency of TYROBP in cerebral Aβ amyloidosis mouse normalizes clinical phenotype and complement subnetwork molecular pathology without reducing Aβ burden. *Mol. Psychiatry* 24, 431–446. doi: 10.1038/s41380-018-0255-6

Hawrylycz, M. J., Lein, E. S., Guillozet-Bongaarts, A. L., Shen, E. H., Ng, L., Miller, J. A., et al. (2012). An anatomically comprehensive atlas of the adult human brain transcriptome. *Nature* 489, 391–399. doi: 10.1038/nature11405

Heard, E., and Martienssen, R. A. (2014). Transgenerational epigenetic inheritance: myths and mechanisms. *Cell* 157, 95–109. doi: 10.1016/j.cell.2014.02.045

Hinde, C. A., and Kilner, R. M. (2007). Negotiations within the family over the supply of parental care. *Proc. R. Soc. B Biol. Sci.* 274, 53–60. doi: 10.1098/rspb.2006.3692

Hiyama, G., Kansaku, N., Tanaka, T., Wakui, S., and Zadworny, D. (2015). Characterization of chicken prolactin regulatory element binding protein and its expression in the anterior pituitary gland during embryogenesis and different reproductive stages. *J. Poult. Sci.* 52, 42–51. doi: 10.2141/jpsa.0140036

Jablonka, E., and Raz, G. (2009). Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. *Q. Rev. Biol.* 84, 131–176.

Jimeno, B., Hau, M., Gomez-Diaz, E., and Verhulst, S. (2019). Developmental conditions modulate DNA methylation at the glucocorticoid receptor gene with cascading effects on expression and corticosterone levels in zebra finches. *Sci. Rep.* 9:11. doi: 10.1038/s41598-019-52203-8

Johnsson, M., Jonsson, K. B., Andersson, L., Jensen, P., and Wright, D. (2015). Genetic regulation of bone metabolism in the chicken: similarities and differences to mammalian systems. *PLoS Genet.* 11:e1005250. doi: 10.1371/journal.pgen.1005250

Kahn, H. S., Graff, M., Stein, A. D., and Lumey, L. H. (2009). A fingerprint marker from early gestation associated with diabetes in middle age: the dutch hunger winter families study. *Int. J. Epidemiol.* 38, 101–109. doi: 10.1093/ije/dyn158

Kappeler, L., and Meaney, M. J. (2010). Epigenetics and parental effects. *BioEssays* 32, 818–827. doi: 10.1002/bies.201000015

Keller, L. F., and van Noordwijk, A. J. (1994). Effects of local environmental conditions on nestling growth in the great tit *Parus major* L. *Ardea* 82, 349–362.

Kesherwani, V., Shahshahan, H. R., and Mishra, P. K. (2017). Cardiac transcriptome profiling of diabetic Akita mice using microarray and next generation sequencing. *PLoS One* 12:e0182828. doi: 10.1371/journal.pone.0182828

Khoonsari, P. E., Haggmark, A., Lonnberg, M., Mikus, M., Kilander, L., Lannfelt, L., et al. (2016). Analysis of the cerebrospinal fluid proteome in Alzheimer's disease. *PLoS One* 11:e0150672. doi: 10.1371/journal.pone.0150672

Kilvitis, H. J., Alvarez, M., Foust, C. M., Schrey, A. W., Robertson, M., and Richards, C. L. (2014). "Ecological epigenetics," in *Ecological Genomics: Ecology and*

the Evolution of Genes and Genomes Advances in Experimental Medicine and Biology, eds C. R. Landry and N. AubinHorth (New York: Springer), 191–210.

Krause, E. T., Honarmand, M., Wetzel, J., and Naguib, M. (2009). Early fasting is long lasting: differences in early nutritional conditions reappear under stressful conditions in adult female zebra finches. *PLoS One* 4:e5015. doi: 10.1371/journal.pone.0005015

Krueger, F. (2012). *Trim Galore: A Wrapper Tool Around Cutadapt and FastQC to Consistently Apply Quality and Adapter Trimming to FastQ files, with Some Extra Functionality for MspI-digested RRBS-type (Reduced Representation Bisufite-Seq) Libraries*. Available online at: https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/ (accessed October 7, 2019).

Laine, V. N., Gossmann, T. I., Schachtschneider, K. M., Garroway, C. J., Madsen, O., Verhoeven, K. J. F., et al. (2016). Evolutionary signals of selection on cognition from the great tit genome and methylome. *Nat. Commun.* 7:9. doi: 10.1038/ncomms10474

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:10. doi: 10.1186/gb-2009-10-3-r25

Lawrence, M., Gentleman, R., and Carey, V. (2009). rtracklayer: an R package for interfacing with genome browsers. *Bioinformatics* 25, 1841–1842. doi: 10.1093/bioinformatics/btp328

Lawrence, M., Huber, W., Pagès, H., Aboyoun, P., Carlson, M., Gentleman, R., et al. (2013). Software for computing and annotating genomic ranges. *PLoS Comput. Biol.* 9:e1003118. doi: 10.1371/journal.pcbi.1003118

Lindner, M., Laine, V. N., Verhagen, I., Viitaniemi, H. M., Visser, M. E., van Oers, K., et al. (2021a). Rapid changes in DNA methylation associated with the initiation of reproduction in a small songbird. *Mol. Ecol.* doi: 10.1111/mec.15803 [Epub ahead of print].

Lindner, M., Verhagen, I., Viitaniemi, H. M., Laine, V. N., Visser, M. E., van Oers, K., et al. (2021b). Temporal changes in DNA methylation and RNA expression in a small song bird: within- and between-tissue comparisons. *BMC Genomics* 22:36. doi: 10.1186/s12864-020-07329-9

Liu, L., Yang, N., Xu, G. Y., Liu, S. L., Wang, D., Song, J. Z., et al. (2018). Transgenerational transmission of maternal stimulatory experience in domesticated birds. *FASEB J.* 32, 7002–7017. doi: 10.1096/fj.201800762RR

Mathot, K. J., Olsen, A.-L., Mutzel, A., Araya-Ajoy, Y. G., Nicolaus, M., Westneat, D. F., et al. (2017). Provisioning tactics of great tits (Parus major) in response to long-term brood size manipulations differ across years. *Behav. Ecol.* 28, 1402–1413. doi: 10.1093/beheco/arx083

McGhee, K. E., and Bell, A. M. (2014). Paternal care in a fish: epigenetics and fitness enhancing effects on offspring anxiety. *Proc. R. Soc. B Biol. Sci.* 281;6. doi: 10.1098/rspb.2014.1146

Meissner, A. (2005). Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. *Nucleic Acids Res.* 33, 5868–5877. doi: 10.1093/nar/gki901

Mertens, J. A. L. (1969). Influence of brood size on energy metabolism and water loss of nestling great tits *Parus major* major. *IBIS* 111, 11–18. doi: 10.1111/j.1474-919X.1969.tb01599.x

Moore, L. D., Le, T., and Fan, G. (2013). DNA methylation and its basic function. *Neuropsychopharmacology* 38, 23–38. doi: 10.1038/npp.2012.112

Mutzel, A., Dingemanse, N. J., Araya-Ajoy, Y. G., and Kempenaers, B. (2013). Parental provisioning behaviour plays a key role in linking personality with reproductive success. *Proc. R. Soc. B Biol. Sci.* 280:9. doi: 10.1098/rspb.2013.1019

Naef-Daenzer, B., and Keller, L. F. (1999). The foraging performance of great and blue tits (Parus major and P-caerulens) in relation to caterpillar development, and its consequences for nestling growth and fledging weight. *J. Anim. Ecol.* 68, 708–718. doi: 10.1046/j.1365-2656.1999.00318.x

Naguib, M., Florcke, C., and van Oers, K. (2011). Effects of social conditions during early development on stress response and personality traits in great tits (*Parus major*). *Dev. Psychobiol.* 53, 592–600. doi: 10.1002/dev.20533

Naguib, M., Nemitz, A., and Gil, D. (2006). Maternal developmental stress reduces reproductive success of female offspring in zebra finches. *Proc. R. Soc. B Biol. Sci.* 273, 1901–1905. doi: 10.1098/rspb.2006.3526

Naguib, M., Riebel, K., Marzal, A., and Gil, D. (2004). Nestling immunocompetence and testosterone covary with brood size in a songbird. *Proc. R. Soc. Lond. B Biol. Sci.* 271, 833–838. doi: 10.1098/rspb.2003.2673

Natt, D., Agnvall, B., and Jensen, P. (2014). Large sex differences in chicken behavior and brain gene expression coincide with few differences in promoter DNA-methylation. *PLoS One* 9:e0096376. doi: 10.1371/journal.pone.0096376

Nettle, D., Andrews, C. P., Monaghan, P., Brilot, B. O., Bedford, T., Gillespie, R., et al. (2015). Developmental and familial predictors of adult cognitive traits in the European starling. *Anim. Behav.* 107, 239–248. doi: 10.1016/j.anbehav.2015.07.002

Nettle, D., Monaghan, P., Boner, W., Gillespie, R., and Bateson, M. (2013). Bottom of the heap: having heavier competitors accelerates early-life telomere loss in the European starling, *Sturnus vulgaris*. *PLoS One* 8:e0083617. doi: 10.1371/journal.pone.0083617

Neuenschwander, S., Brinkhof, M. W. G., Kolliker, M., and Richner, H. (2003). Brood size, sibling competition, and the cost of begging in great tits (*Parus major*). *Behav. Ecol.* 14, 457–462. doi: 10.1093/beheco/arg025

Park, J. M., Kim, M. Y., Kim, T. H., Min, D. K., Yang, G. E., and Ahn, Y. H. (2018). Prolactin regulatory element-binding (PREB) protein regulates hepatic glucose homeostasis. *Biochim. Biophys. Acta Mol. Basis Dis.* 1864, 2097–2107. doi: 10.1016/j.bbadis.2018.03.024

Perrins, C. M., and McCleery, R. H. (1989). Laying dates and clutch size in the great tit. *Wilson Bull.* 101, 236–253.

Pertille, F., Brantsaeter, M., Nordgreen, J., Coutinho, L. L., Janczak, A. M., Jensen, P., et al. (2017). DNA methylation profiles in red blood cells of adult hens correlate with their rearing conditions. *J. Exp. Biol.* 220, 3579–3587. doi: 10.1242/jeb.157891

Pettifor, R. A., Perrins, C. M., and McCleery, R. H. (1988). Individual optimization of clutch size in Great Tits. *Nature* 336, 160–162. doi: 10.1038/336160a0

Pettifor, R. A., Perrins, C. M., and McCleery, R. H. (2001). The individual optimization of fitness: variation in reproductive output, including clutch size, mean nestling mass and offspring recruitment, in manipulated broods of great tits *Parus major*. *J. Anim. Ecol.* 70, 62–79. doi: 10.1046/j.1365-2656.2001.00465.x

Reddon, A. R. (2012). Parental effects on animal personality. *Behav. Ecol.* 23, 242–245. doi: 10.1093/beheco/arr210

Reyna-López, G. E., Simpson, J., and Ruiz-Herrera, J. (1997). Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. *Mol. Gen. Genet. MGG* 253, 703–710. doi: 10.1007/s004380050374

Riyahi, S., Sanchez-Delgado, M., Calafell, F., Monk, D., and Senar, J. C. (2015). Combined epigenetic and intraspecific variation of the DRD4 and SERT genes influence novelty seeking behavior in great tit *Parus major*. *Epigenetics* 10, 516–525. doi: 10.1080/15592294.2015.1046027

Roseboom, T., de Rooij, S., and Painter, R. (2006). The Dutch famine and its long-term consequences for adult health. *Early Hum. Dev.* 82, 485–491. doi: 10.1016/j.earlhumdev.2006.07.001

Roulin, A., Emaresi, G., Bize, P., Gasparini, J., Piault, R., and Ducrest, A.-L. (2011). Pale and dark reddish melanic tawny owls differentially regulate the level of blood circulating POMC prohormone in relation to environmental conditions. *Oecologia* 166, 913–921. doi: 10.1007/s00442-011-1955-7

Rubenstein, D. R., Skolnik, H., Berrio, A., Champagne, F. A., Phelps, S., and Solomon, J. (2016). Sex-specific fitness effects of unpredictable early life conditions are associated with DNA methylation in the avian glucocorticoid receptor. *Mol. Ecol.* 25, 1714–1728. doi: 10.1111/mec.13483

Saino, N., Suffritti, C., Martinelli, R., Rubolini, D., and Møller, A. P. (2003). Immune response covaries with corticosterone plasma levels under experimentally stressful conditions in nestling barn swallows (*Hirundo rustica*). *Behav. Ecol.* 14, 318–325. doi: 10.1093/beheco/14.3.318

Sajuthi, S. P., Sharma, N. K., Chou, J. W., Palmer, N. D., McWilliams, D. R., Beal, J., et al. (2016). Mapping adipose and muscle tissue expression quantitative trait loci in African Americans to identify genes for type 2 diabetes and obesity. *Hum. Genet.* 135, 869–880. doi: 10.1007/s00439-016-1680-8

Sanz, J. J., and Tinbergen, J. M. (1999). Energy expenditure, nestling age, and brood size: an experimental study of parental behavior in the great tit *Parus major*. *Behav. Ecol.* 10, 598–606. doi: 10.1093/beheco/10.5.598

Schrey, A. W., Alvarez, M., Foust, C. M., Kilvitis, H. J., Lee, J. D., Liebl, A. L., et al. (2013). Ecological epigenetics: beyond MS-AFLP. *Integr. Comp. Biol.* 53, 340–350. doi: 10.1093/icb/ict012

Schulz, L. C. (2010). The Dutch Hunger Winter and the developmental origins of health and disease. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16757–16758. doi: 10.1073/pnas.1012911107

Schwabl, H. (1993). Yolk is a source of maternal testosterone for developing birds. *Proc. Natl. Acad. Sci. U.S.A.* 90, 11446–11450. doi: 10.1073/pnas.90.24.11446

Sepers, B., van den Heuvel, K., Lindner, M., Viitaniemi, H., Husby, A., and van Oers, K. (2019). Avian ecological epigenetics: pitfalls and promises. *J. Ornithol.* 160, 1183–1203. doi: 10.1007/s10336-019-01684-5

Sheldon, E. L., Schrey, A. W., Ragsdale, A. K., and Griffith, S. C. (2018). Brood size influences patterns of DNA methylation in wild Zebra Finches (*Taeniopygia guttata*). *Auk* 135, 1113–1122. doi: 10.1642/auk-18-61.1

Singh, R. D., Schroeder, A. S., Scheffer, L., Holicky, E. L., Wheatley, C. L., Marks, D. L., et al. (2013). Prominin-2 expression increases protrusions, decreases caveolae and inhibits Cdc42 dependent fluid phase endocytosis. *Biochem. Biophys. Res. Commun.* 434, 466–472. doi: 10.1016/j.bbrc.2013.03.097

Son, W. Y., Hwang, S. H., Han, C. T., Lee, J. H., Kim, S., and Kim, Y. C. (1999). Specific expression of heat shock protein HspA2 in human male germ cells. *Mol. Hum. Reprod.* 5, 1122–1126. doi: 10.1093/molehr/5.12.1122

Spurgin, L. G., Bosse, M., Adriaensen, F., Albayrak, T., Barboutis, C., Belda, E., et al. (2019). The great tit HapMap project: a continental-scale analysis of genomic variation in a songbird. *bioRxiv* [Preprint]. doi: 10.1101/561399

Stanner, S. A., and Yudkin, J. S. (2001). Fetal programming and the leningrad siege study. *Twin Res.* 4, 287–292. doi: 10.1375/twin.4.5.287

St-Cyr, S., and McGowan, P. O. (2015). Programming of stress-related behavior and epigenetic neural gene regulation in mice offspring through maternal exposure to predator odor. *Front. Behav. Neurosci.* 9:145. doi: 10.3389/fnbeh.2015.00145

Szwergold, B. S., Bunker, R. D., and Loomes, K. M. (2011). The physiological substrates of fructosamine-3-kinase-related-protein (FN3KRP) are intermediates of nonenzymatic reactions between biological amines and ketose sugars (fructation products). *Med. Hypotheses* 77, 739–744. doi: 10.1016/j.mehy.2011.07.027

Tinbergen, J. M., and Boerlijst, M. C. (1990). Nestling weight and survival in individual Great Tits (*Parus major*). *J. Anim. Ecol.* 59, 1113–1127. doi: 10.2307/5035

Tobi, E. W., Goeman, J. J., Monajemi, R., Gu, H., Putter, H., Zhang, Y., et al. (2014). DNA methylation signatures link prenatal famine exposure to growth and metabolism. *Nat. Commun.* 5:5592. doi: 10.1038/ncomms6592

Tobi, E. W., Lumey, L. H., Talens, R. P., Kremer, D., Putter, H., Stein, A. D., et al. (2009). DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. *Hum. Mol. Genet.* 18, 4046–4053. doi: 10.1093/hmg/ddp353

Trent, S., Fry, J. P., Ojarikre, O. A., and Davies, W. (2014). Altered brain gene expression but not steroid biochemistry in a genetic mouse model of neurodevelopmental disorder. *Mol. Autism* 5:21. doi: 10.1186/2040-2392-5-21

van Oers, K., Drent, P. J., Dingemanse, N. J., and Kempenaers, B. (2008). Personality is associated with extrapair paternity in great tits, *Parus major*. *Anim. Behav.* 76, 555–563. doi: 10.1016/j.anbehav.2008.03.011

van Oers, K., Kohn, G. M., Hinde, C. A., and Naguib, M. (2015). Parental food provisioning is related to nestling stress response in wild great tit nestlings: implications for the development of personality. *Front. Zool.* 12:10. doi: 10.1186/1742-9994-12-s1-s10

van Oers, K., Sepers, B., Sies, W., Gawehns, F., Verhoeven, K. J. F., and Laine, V. N. (2020). Epigenetics of animal personality: DNA methylation cannot explain the heritability of exploratory behavior in a songbird. *Integr. Comp. Biol.* 60, 1517–1530. doi: 10.1093/icb/icaa138

Verhulst, E. C., Mateman, A. C., Zwier, M. V., Caro, S. P., Verhoeven, K. J. F., and Van Oers, K. (2016). Natural variation in animal personality is associated with transgenerationally consistent DNA methylation. *Mol. Ecol.* 25, 1801–1811. doi: 10.1111/mec.13519

Verhulst, S., Holveck, M.-J., and Riebel, K. (2006). Long-term effects of manipulated natal brood size on metabolic rate in zebra finches. *Biol. Lett.* 2, 478–480. doi: 10.1098/rsbl.2006.0496

Viitaniemi, H. M., Verhagen, I., Visser, M. E., Honkela, A., van Oers, K., and Husby, A. (2019). Seasonal variation in genome-wide DNA methylation patterns and the onset of seasonal timing of reproduction in great tits. *Genome Biol. Evol.* 11, 970–983. doi: 10.1093/gbe/evz044

Wang, S. H., Cheng, C. Y., Tang, P. C., Chen, C. F., Chen, H. H., Lee, Y. P., et al. (2013). Differential gene expressions in testes of L2 strain Taiwan country chicken in response to acute heat stress. *Theriogenology* 79, 374–382. doi: 10.1016/j.theriogenology.2012.10.010

Wang, S. H., Cheng, C. Y., Tang, P. C., Chen, C. F., Chen, H. H., Lee, Y. P., et al. (2015). Acute heat stress induces differential gene expressions in the testes of a broiler-type strain of taiwan country chickens. *PLoS One* 10:e0125816. doi: 10.1371/journal.pone.0125816

Weaver, I. C. G., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., et al. (2004). Epigenetic programming by maternal behavior. *Nat. Neurosci.* 7, 847–854. doi: 10.1038/nn1276

Wilkin, T. A., King, L. E., and Sheldon, B. C. (2009). Habitat quality, nestling diet, and provisioning behaviour in great tits *Parus major*. *J. Avian Biol.* 40, 135–145. doi: 10.1111/j.1600-048X.2009.04362.x

Willson, N. L., Forder, R. E. A., Tearle, R., Williams, J. L., Hughes, R. J., Nattrass, G. S., et al. (2018). Transcriptional analysis of liver from chickens with fast (meat bird), moderate (F1 layer x meat bird cross) and low (layer bird) growth potential. *BMC Genomics* 19:12. doi: 10.1186/s12864-018-4723-9

Wingett, S. W., and Andrews, S. (2018). FastQ screen: a tool for multi-genome mapping and quality control. *F1000Research* 7:1338. doi: 10.12688/f1000research.15931.2

Wright, J., Both, C., Cotton, P. A., and Bryant, D. (1998). Quality vs. quantity: energetic and nutritional trade-offs in parental provisioning strategies. *J. Anim. Ecol.* 67, 620–634.

Zeng, H. K., Shen, E. H., Hohmann, J. G., Oh, S. W., Bernard, A., Royall, J. J., et al. (2012). Large-scale cellular-resolution gene profiling in human neocortex reveals species-specific molecular signatures. *Cell* 149, 483–496. doi: 10.1016/j.cell.2012.02.052

Zhu, J., Wang, Y., Zhu, K., Gao, J., Wan, X., Pang, X., et al. (2017). Serum fibroblast growth factor 1 is associated with the decreased risk of obesity in human. *Exp. Clin. Endocrinol. Diabetes* 125, 322–326. doi: 10.1055/s-0043-104532

Zimmer, C., Larriva, M., Boogert, N. J., and Spencer, K. A. (2017). Transgenerational transmission of a stress-coping phenotype programmed by early-life stress in the Japanese quail. *Sci. Rep.* 7:19. doi: 10.1038/srep46125

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Pipefish Locally Adapted to Low Salinity in the Baltic Sea Retain Phenotypic Plasticity to Cope With Ancestral Salinity Levels

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Genetic adaptation and phenotypic plasticity facilitate the migration into new habitats and enable organisms to cope with a rapidly changing environment. In contrast to genetic adaptation that spans multiple generations as an evolutionary process, phenotypic plasticity allows acclimation within the life-time of an organism. Genetic adaptation and phenotypic plasticity are usually studied in isolation, however, only by including their interactive impact, we can understand acclimation and adaptation in nature. We aimed to explore the contribution of adaptation and plasticity in coping with an abiotic (salinity) and a biotic (*Vibrio* bacteria) stressor using six different populations of the broad-nosed pipefish *Syngnathus typhle* that originated from either high [14–17 Practical Salinity Unit (PSU)] or low (7–11 PSU) saline environments along the German coastline of the Baltic Sea. We exposed wild caught animals, to either high (15 PSU) or low (7 PSU) salinity, representing native and novel salinity conditions and allowed animals to mate. After male pregnancy, offspring was split and each half was exposed to one of the two salinities and infected with *Vibrio alginolyticus* bacteria that were evolved at either of the two salinities in a fully reciprocal design. We investigated life-history traits of fathers and expression of 47 target genes in mothers and offspring. Pregnant males originating from high salinity exposed to low salinity were highly susceptible to opportunistic fungi infections resulting in decreased offspring size and number. In contrast, no signs of fungal infection were identified in fathers originating from low saline conditions suggesting that genetic adaptation has the potential to overcome the challenges encountered at low salinity. Offspring from parents with low saline origin survived better at low salinity suggesting genetic adaptation to low salinity. In addition, gene expression analyses of juveniles indicated patterns of local adaptation, *trans*-generational plasticity and developmental plasticity. In conclusion, our study suggests that pipefish are locally adapted to the low salinity in their environment, however, they are retaining phenotypic plasticity, which allows them to also cope with ancestral salinity levels and prevailing pathogens.

Keywords: *trans*-generational plasticity, genetic adaptation, local adaptation, phenotypic plasticity, Baltic Sea, climate change, salinity, Syngnathids

INTRODUCTION

Genetic adaptation and phenotypic plasticity (Chevin et al., 2010) facilitate the migration of organisms into new habitats and permit coping with changing environmental conditions (Brierley and Kingsford, 2009; Poloczanska et al., 2013; Urban, 2015). Genetic adaptation is a multigenerational evolutionary process spreading in a population over the rise and fixation of novel mutations (Chatterjee et al., 2014), or over selection on standing genetic variation and shifts in allele frequency (Barrett and Schluter, 2008; Eizaguirre et al., 2012; Torda et al., 2017). In contrast, phenotypic plasticity is an individual trait that enables organisms of one genotype to show multiple, alternative phenotypes in response to biotic or abiotic conditions (West-Eberhard, 1989). The environment influences the phenotype (Chevin et al., 2010) and elicits changes in gene expression, which have the ability to impact individual development, morphology, physiology, and behavior (Angers et al., 2010). Phenotypic responses occur within the life-time of an organism (reversible and developmental plasticity) and can persist across one or several generations (*trans*-generational plasticity) (Sunday et al., 2014).

Trans-generational plasticity (TGP) is the non-genetic inheritance of an alternative phenotype by transferring nutrients, hormones, proteins, or epigenetic marks from the parent to the offspring generation (Sunday et al., 2014). The impact of TGP may differ among species, life stages and abiotic conditions (Uller et al., 2013; Laland et al., 2014) as well as the biotic interaction partners (e.g., parasite type or strain) (Beemelmanns and Roth, 2016a,b, 2017; Roth et al., 2018). TGP can be adaptive and result in increased offspring performance when environmental conditions of parental and offspring generations match (Sunday et al., 2014). This has been shown for instance in wild Atlantic silversides exposed to ocean acidification (Murray et al., 2014) or in three-spined sticklebacks exposed to heat stress (Shama and Wegner, 2014). However, TGP can also induce negative carry-over effects, i.e., if parents are exposed to challenging conditions, juveniles may be negatively affected by the transfer of stress hormones from the parents to the eggs or adult allocate resources in their own survival rather than in the fitness of their offspring (Eriksen et al., 2006; Marshall, 2008). For example, mortality increased in the early life stages of sticklebacks upon changes in salinity levels of the parental generation (Heckwolf et al., 2018).

On an evolutionary time scale, phenotypic plasticity was suggested to slow down genetic adaptation by buffering the effects of natural selection (Kelly, 2019). However, the specific impact of phenotypic plasticity on genetic adaptation is driven by various factors including the species, the traits assessed and the level of current environmental variability and predictability (Reed et al., 2010). The outcome of the interacting genetic adaptation and phenotypic plasticity on the adaptation toward environmental change is thus still debated, as it has been rarely addressed. Instead, the two processes have been mainly studied in isolation (Gienapp et al., 2008; Lind et al., 2020). To depict and understand biological responses to environmental change, we need models (Donelson et al., 2019) and experiments (Kelly, 2019) addressing these processes simultaneously. An approach to study the interaction between genetic adaptations and phenotypic plasticity are space-for-time experiments (Blois et al., 2013; Kelly, 2019), where organisms living along a natural environmental gradient can serve as a prediction for how organisms can cope with future environmental conditions (Reusch et al., 2018).

The main focus of climate change research relies on warming and ocean acidification (but see DeFaveri and Merila, 2014; Hasan et al., 2017; Heckwolf et al., 2018). It is often neglected that changing ocean salinities (Meier et al., 2006; Andersson et al., 2015; Kniebusch et al., 2019) will have strong impacts on coastal and polar ecosystem (Gibson and Najjar, 2000; Loder et al., 2015) by impacting the physiology, metabolism, growth development, immunity and reproduction of marine organisms (Morgan and Iwama, 1991; Haddy and Pankhurst, 2000; Boeuf and Payan, 2001; Velasco et al., 2019), among them teleost fishes like the common goby (Mück and Heubel, 2018), turbot (Nissling et al., 2006), cod (Nissling and Westin, 1997) and flounder (Ustups et al., 2013; Nissling et al., 2017). Teleosts can cope with distinct salinity levels over a costly adjustment of their osmoregulation, which consumes up to 50% of the total energy budget (Boeuf and Payan, 2001) and results in metabolic trade-offs (DeWitt et al., 1998). Strong selection is thus expected to drive the adaptation toward novel salinities.

The Baltic Sea is particularly prone to future reductions in salinity due to little water exchange with the North Sea and river runoffs from the surrounding countries. Increased precipitation in the northern part may cause a decrease by up to 30% in surface salinity by the end of the century (Meier et al., 2006; Andersson et al., 2015). Already today, the Baltic Sea is characterized by a strong salinity gradient ranging from 30 PSU in the transition to the North Sea to an almost freshwater environment in the north-eastern parts making it an ideal setting for space for time experiments (Blanquart and Gandon, 2013; Heckwolf et al., 2018). The stability of the salinity gradient (Janssen et al., 1999; Hinrichs et al., 2019), the energetic cost of both, osmoregulation (Boeuf and Payan, 2001), and phenotypic plasticity (DeWitt et al., 1998), promote genetic adaptation in teleost fishes toward different salinity levels in the Baltic Sea (DeFaveri and Merila, 2014; Berg et al., 2015; Guo et al., 2015, 2016). In relatively stable new salinity environments, genetic assimilation was suggested to result in reduced plasticity and more adaptive genotypes (Angers et al., 2010). Adaptation to the low salinity conditions of the Baltic Sea and the isolation from the Atlantic source population is also accompanied by a loss of genetic diversity (Johannesson and Andre, 2006; Holmborn et al., 2011). Therefore, adaptation to low salinity can result in reduced osmoregulatory plasticity, such as changes in kidney morphology and gene expression (Hasan et al., 2017), and thus hamper the ability to cope with further salinity fluctuations. TGP was predicted to not be sufficient to buffer the negative impacts of salinity change (Heckwolf et al., 2018), in particular if salinity is subject to strong fluctuations and if populations are locally adapted. In contrast, increased selection due to negative carry-over effects may facilitate rapid adaptation (Heckwolf et al., 2018).

A suitable organism to study the interactive contribution of genetic adaptation and phenotypic plasticity is the broad-nosed pipefish *Syngnathus typhle* (Syngnathidae, Teleostei) (Wilson et al., 2020). *S. typhle* inhabits a wide range of waters with

different salinity levels along the European coastline from the Black Sea in Eastern Europe to the Mediterranean Sea and the Eastern Atlantic (Wilson and Veraguth, 2010). After the last glacial maximum and thus in less than 8000 years, the pipefish migrated from the full marine salinity environment of the North Sea and Atlantic (33–35 PSU) into the brackish Baltic Sea with relatively low salinity levels (Björck, 1995; Wilson and Veraguth, 2010). TGP in response to immune and temperature challenges has been demonstrated in broad-nosed pipefish in numerous studies (Beemelmanns and Roth, 2016b, 2017; Roth and Landis, 2017) as well as the impairing effect of low salinity on the immune system (Birrer et al., 2012). Beyond the direct impact of salinity changes on organisms and populations (genotype × environment interaction, G×E), salinity shifts may increase or decrease the virulence of parasites and pathogens (genotype × genotype × environment interaction, G×G×E) (Stockwell et al., 2011; Hall et al., 2013; Poirier et al., 2017) and alter co-evolutionary dynamics between host and pathogens (Mostowy and Engelstadter, 2011; Molnar et al., 2013; Brunner and Eizaguirre, 2016; Kutzer and Armitage, 2016).

The abundance and virulence of opportunistic and omnipresent marine pathogens, such as several strains of the *Vibrio* bacteria clade (Baker-Austin et al., 2017) are modulated by salinity and temperature (Chen et al., 2011; Oberbeckmann et al., 2011; Baker-Austin et al., 2017). *Vibrio alginolyticus* frequently infects pipefish in the Baltic Sea (Roth et al., 2012a) and is known to cause higher mortality in artemia and herring at low salinity (Dayma et al., 2015; Poirier et al., 2017). Increases in bacterial virulence are evoked due to a combination of phenotypic changes, including bacterial biofilm formation (Dayma et al., 2015; Kim and Chong, 2017) and the expression of bacterial motility and virulence factors (Hase and Barquera, 2001; Wendling et al., 2017). We hypothesized that genetic adaptation of the pipefish to local salinity and the prevailing pathogens may compensate for the previously observed drop of immunological activity in case of exposure to decreasing salinities (Birrer et al., 2012; Poirier et al., 2017) and, hence, has the potential to reduce the negative impact of pathogens like *Vibrio* bacteria (Roth et al., 2012a).

To explore how the pipefish *S. typhle* has genetically adapted to long-term salinity changes and how this adaptation influences its phenotypic plasticity in coping with short-term shifts in salinity, we compared the potential of pipefish originating from relatively high and relatively low salinity environments in the Baltic Sea to react toward salinity shifts (Meier et al., 2006; Kniebusch et al., 2019) with developmental and *trans*-generational plasticity. Furthermore, we investigated how adaptation and acclimation of the pipefish host and the bacterial *Vibrio* pathogen to high and low salinity changes the host-pathogen interaction. We tested the following hypotheses: (1) *S. typhle* populations are genetically adapted to the salinity in their local habitat, (2) adaptive *trans*-generational plasticity in matching parental and offspring salinity results in enhanced juvenile survival and matching gene expression pattern in the parental and offspring generation, (3) *S. typhle* populations locally adapted to low salinity have reduced phenotypic plasticity and are not able to cope with ancestral salinity levels, and (4) bacterial virulence is higher at low salinity.

To investigate how *S. typhle* have adapted toward their local salinity and local pathogens in the past (genetic adaptation) and to assess their consecutive acclimation potential (phenotypic plasticity) toward salinity shifts and their immune response toward a bacterial infection, we collected six *S. typhle* populations in the Baltic Sea. Fish were collected at three sampling sites with high saline conditions and at three sampling sites with low saline conditions. In a laboratory aquaria experiment, animals were exposed to either their native salinity (high or low, respectively) or the salinity of the other three populations (novel salinity). Upon successful male pregnancy, offspring were exposed to either native or novel salinity conditions, in a fully reciprocal design. Subsequently, juvenile fish were injected with a *V. alginolyticus* strain that evolved for 90 days either at low or high salinity in the laboratory. In addition to life-history traits and mortality, we investigated the expression of 47 target genes involved in (i) general metabolism, (ii) immune response, (iii) gene regulation (DNA and histone modification), and (iv) osmoregulation.

MATERIALS AND METHODS

Sampling of Adult Pipefish Populations

The parental *Syngnathus typhle* generation (females and non-pregnant males) was caught in seagrass meadows of six sampling sites along the German coastline of the Baltic Sea in spring 2017 before the onset of the reproductive season (**Figure 1** and **Table 1**). Three sampling sites are characterized by relatively high salinity conditions (14–17 PSU; high *origin salinity*; *H*) and three sampling sites by relatively low salinity conditions (7–11 PSU; low *origin salinity*, *L*; **Table 1**). Salzhaff was assigned to the category low because salinity drops are common after rainfall accompanied with freshwater discharge due to enclosed morphology of the inlet. Therefore, pipefish in Salzhaff are often exposed to salinity levels below 10 PSU. Pipefish collected in high *origin salinity* are predicted to experience salinity levels of pipefish collected in low *origin salinity* considering the predicted decrease of Baltic Sea surface salinity (Meier et al., 2006; Kniebusch et al., 2019) and short term salinity fluctuations of about 5 PSU below and above the average (Bock and Lieberum, 2017). For the experiment, the six sample sites were assigned to two replicated salinity categories with one above and one below the isosmotic point of approximately 12 PSU. A minimum of 30 non-pregnant males and 30 females were caught snorkeling with hand nets at each sampling site at depths ranging between 0.5 and 2.5 m. At each sampling site water temperature and salinity were measured from water collected about 1 m below the surface using a salinometer (WTW Cond 330i). Pipefish (females and non-pregnant males) were transported in large aerated coolers from the sampling site to the aquaria facilities of the GEOMAR (Westshore) in Kiel (Germany). Females and non-pregnant males were kept separately to avoid mating prior to the experiment. Females of each sampling site were split into two groups and placed into twelve 80-liter tanks with 14 to 16 individuals per aquarium. Males of each sampling site were split into four and placed in total into 24 80-liter with seven to eight individuals per aquarium. These 36 tanks were connected

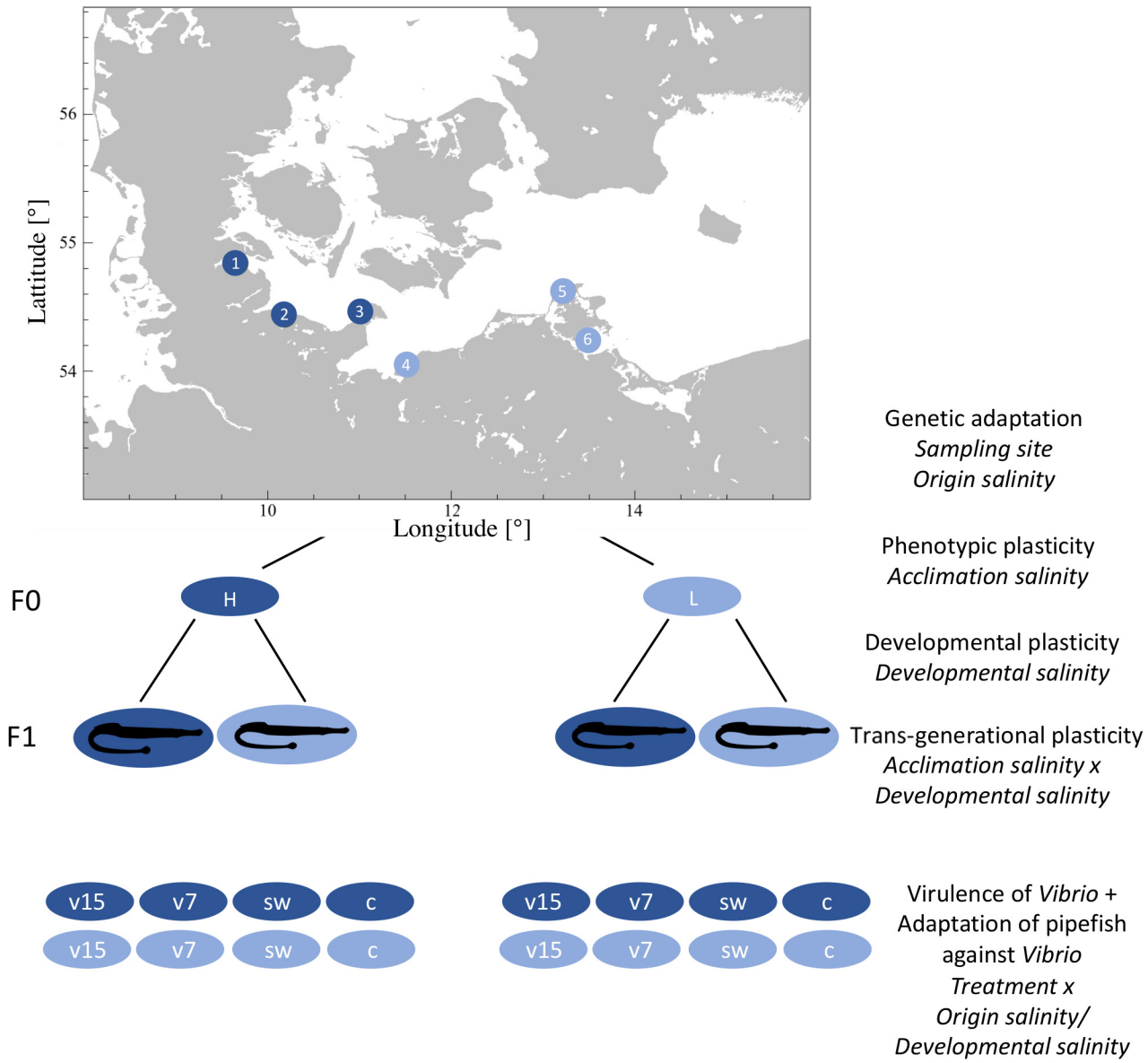

FIGURE 1 | Fully reciprocal experimental design. We sampled pipefish along the Baltic Sea coast, at three *sampling sites* from a relatively high saline environment (high *origin salinity*: 14–17 PSU; dark blue circles; subsequently labeled as italic *H*): (1) Flensburg Fjord, (2) Falckenstein Strand and (3) Fehmarn and three *sampling sites* with a relatively low salinity level (low *origin salinity*: 7–11 PSU; light blue circles; subsequently labeled as italic *L*): (4) the Salzhaff and (5) Ruegen North and (6) Ruegen South. In the laboratory males and females were kept separately and acclimated to the opposing salinity [*acclimation salinity*: 15 PSU (H, dark blue), 7 PSU (L, light blue)] or their respective native salinity. Subsequently, males and females were allowed to mate and pregnant males were kept at constant conditions. Half of the F1 generation was either exposed to high (h) or low (l) salinity within 24 h after birth (*developmental salinity*). Ten days post hatch juveniles were injected with *Vibrio alginolyticus* evolved at 15 PSU (v15) or at 7 PSU (v7), sham injected with sterile seawater (sw) or left naive (c) (*treatment*). Label in italic on the right side correspond to the factors that were considered in the statistical models.

to two independent circulating water systems containing either high saline (15 PSU; $n = 18$) or low saline water (7 PSU, $n = 18$) and equipped with artificial seagrass. Pipefish from high *origins salinity* were kept at 15 PSU (Baltic Sea water) and those from low *origins salinity* at 7 PSU (Baltic Sea water, diluted with deionized water and tap water (ratio 2:1:1) to keep the water alkalinity constant). The water temperature throughout the experiment was 18°C and illumination was set to a 16:8 h day and night cycle. Pipefish adults were fed twice a day with frozen and occasionally with live mysids.

After pipefish were acclimated to laboratory conditions for at least 2 days, half of the individuals from each sampling site were gradually acclimated to the novel salinity over 4 days. Each day, tanks were briefly connected to the 15 PSU or the 7 PSU circulating system to either increase or decrease the salinity by 1.5 to 2 PSU. The other half of the fish remained in their native

TABLE 1 | Pipefish sampling sites with coordinates, sampling date and ambient salinity and water temperature.

Sampling sites (Abbreviation)	GPS Coordinates	Salinity (PSU)	Salinity (Category)	Water Temperature (°C)
Flensburg Fjord, Westerholz (Flens)	54°49′14 N 9°40′26 E	17	High	15
Kiel Fjord, Falckensteiner Strand (Falck)	54°23′26 N 10°11′33 E	14	High	10–11
Orther Bay, Fehmarn (Fehm)	54°26′55 N 11°3′19 E	15	High	13
Salzhaff, Werder (Salz)	54°1′35 N 11°3′57 E	10–11	Low	14
Wieker Bodden, Wiek (RuegN)	54°37′20 N 13°16′56 E	8	Low	18
Strelasund, Grabow (RuegS)	54°13′32 N 13°24′25 E	7	Low	12

salinity. Apart from the salinity adjustment, all 36 tanks remained disconnected from the circulation system during the time of salinity acclimation.

One day after the final salinity acclimation, four to six randomly chosen males and four to six females originating from the same sampling site and acclimated to the same salinity, were placed together in one of the 36 tanks connected to circulating water systems of either high or low *acclimation salinity* (**Figure 1**). During mating and male pregnancy, fish maintenance and aquaria set-up remained as described.

One week after mating, some pipefish males started to show signs of infection with a fungus growing inside and on the brood pouch. The causative agent could be the oomycete *Saprolegnia*, given prevalent *Saprolegnia* water molds on fish eggs in low saline waters (Lehtonen and Kvarnemo, 2015). Three weeks after mating, we visually assessed and photographically documented the prevalence of the fungus.

Sampling of Adult Pipefish for Targeted Gene Expression, Population Genetics, and Life History Traits

Four days after mating, females were removed from the tanks and immediately euthanized using anesthetic tricaine methane sulfonate (MS-222, 500 mg/L). We measured standard body length and total weight and removed the gills to store them in RNA*later* at 4°C overnight and subsequently at −80°C. Fin clips were taken and placed in 96% Ethanol for population genetic analysis. Standard body length and total weight of males were measured one to three days after males gave birth.

Population Genetics Using Microsatellites

DNA Isolation and Preparation

Genomic DNA was isolated from fin clips of F0 female pipefish using the DNeasy 96 Blood and Tissue Kit (QIAGEN, Venlo, Netherlands) following the manufacturer's protocol. All samples were incubated and eluted twice to obtain a higher extraction yield. A subset of the isolated genomic DNA was quantified using NanoDrop (Spectrometer; Peqlab, Erlangen, Germany) and visually evaluated by gel electrophoresis on a 1.2% agarose gel [GelRed nucleic acid stain, Lambda DNA/HindIII Marker and 1 kb DNA marker (Invitrogen; Thermo Fisher Scientific, Germany)].

All 144 female *S. typhle* samples were genotyped for 11 microsatellite loci, with a minimum of 20 individuals per sampling site. Genotyping was performed in three pooled reactions, each containing 3–4 primer pairs that were designed on an expressed sequence tag (EST) library of *S. typhle* [Pool A: Sy_ty_1, Sy_ty_4, Sy_ty_6, Sy_ty_7; Pool B: Sy_ty_11, Sy_ty_22, Sy_ty_23; Pool C: Sy_ty_16, Sy_ty_17, Sy_ty_21, Sy_ty_24 (Jones et al., 1999; Roth et al., 2012a)]. Microsatellites, the associated primer pairs and the Multiplex PCR protocol can be found in GenBank under accession numbers JQ598279–JQ598290 and in the **Supplementary Material 1**. Primers had an initial concentration of 5 pmol and were color labeled with either Hex green or Fam blue to allow differentiation during fragment analysis. In a 10 μl reaction, several loci were amplified simultaneously from 1 μl of extracted DNA using 5 μl of the Multiplex PCR Master Mix (QIAGEN) and varying amounts of the pooled primer mixes (Pool A: 1.75 μl, Pool B: 0.75 μl, Pool C: 1.5 μl). Three negative controls (ddH_2O) were added onto each 96-well plate.

Capillary electrophoresis and fragment analysis were performed using the 3130xl Genetic Analyzer (Applied Biosystems/Thermo Fisher Scientific). A loading mix containing 8.75 μl HiDi Formamide and 0.25 μl GeneScan 350 ROX dye Size Standard (Applied Biosystems/Thermo Fisher Scientific) was added to 1 μl of each PCR product. Prior to the fragment run, samples were denatured in a thermo cycler for 2 min at 90°C.

Microsatellite Analysis

Raw fragment data were scored using the GeneMarker Genotyping Software (Hulce et al., 2011). The software displays allele frequency panels that identify the alleles for each locus in each sample, thus provides an overview of whether individuals are homozygous or heterozygous for certain alleles at a locus. Additionally, the raw data were screened using the Microsatellite Data Checking Software Micro-Checker (Oosterhout et al., 2004). Micro-Checker identifies genotyping errors caused by non-amplified null-alleles that either appear due

to mutations in the primer binding regions or generally occur in fragment analysis because PCR shows greater efficiency in longer sequences. GENETIX (Belkhir, 2004) was used to describe the level to which the genotype frequency differed from the expected Hardy-Weinberg equilibrium (HWE) frequency by calculating a global F_{ST} value as a correlation of inbreeding in the substructure vs. in the entire population. For completeness, pairwise F_{ST} values were calculated to display distances between pairs of haplotypes and a F_{IS} value was calculated as a correlation of inbreeding vs. random mating within the population. Although GENETIX has a greater statistical power, the population structure within the multi-locus genotype data was further investigated by the STRUCTURE Software for Population Genetics Inference (Pritchard et al., 2000). Based on the Bayesian clustering method, STRUCTURE creates an admixture model, which provides likelihood scores for each individual of belonging to a certain population. The model was tested with varying numbers of expected populations ranging from a minimum of two (high salinity vs. low salinity) to a maximum of six (number of sampling stations). Visualization of the population clustering was performed using the PHYLogeny Inference Package PHYLIP (Felsenstein, 1989). PHYLIP provides a pipeline of programs to randomize comparisons, create randomized trees, which are then assembled to a final phylogeographic tree that is based on the most frequent combinations found within the randomized trees. As the retrieved fragment data did not provide any lineage data that allows to draw conclusions with regard to a common ancestor, we created an unrooted phylogeographic tree.

Candidate Gene Expression of Females

To assess local adaptation to salinity and the potential of *S. typhle* to cope with novel salinity conditions, we selected candidate genes from three different functional categories, i.e., (i) immune response, (ii) metabolism, and (iii) gene regulation (DNA and histone modification) (**Supplementary Table 2**). Immune genes were further subdivided into innate, adaptive and complement system genes and gene regulation genes into activating and silencing genes.

RNA Extraction and Reverse Transcription

RNA was extracted from gill tissue of adult female pipefish that was stabilized in RNAlater using the RNeasy® Universal Tissue kit (QIAGEN, Venlo, Netherlands). Tissue samples were homogenized by adding a 5 mm stainless steel bead into each collection tube and placing them into a homogenizer shaking for two times 30 s at 25 Hertz. Thereafter, we followed the manufacturer's protocol "Purification of Total RNA from Animal Tissues Using Spin Technology." RNA concentration (extraction yield) and purity of the samples were checked by spectrophotometry (NanoDrop ND-1000 Spectrometer; Peqlab, Erlangen, Germany). Protein contamination was quantified using the absorption ratio of 260/280 nm (target > 2.0) and the ratio 260/230 nm (target > 1.8) was used to detect organic contamination. A fixed amount of RNA (300 ng/sample = 50 ng/µl) was then reverse transcribed into cDNA using the QuantiTect Reverse Transcriptase kit (QIAGEN, Venlo, Netherlands).

Preamplification of cDNA and Candidate Gene Expression

For each sample, 1.4 µl target cDNA was pre-amplified with 0.5 µl primer pool mix of all 48 genes (500 nM), 2.5 µl TaqMan PreAmp Master Mix (Applied Biosystems, Waltham, MA, United States) and 0.7 µl H_2O (10 min at 95°C, 14 cycles: 15 s at 95°C followed by 4 min at 60°C). Afterward, the PCR product was diluted 1:10 with low TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8). The sample mix for the 96.96 Dynamic Array™ IFCs chips contained 3.1 µl pre-amplified and diluted PCR product, 3.55 µl SsoFast-EvaGreen Supermix with Low ROX (Bio-Rad Laboratories, Hercules, CA, United States) and 0.37 µl 20 x DNA Binding Dye Sample Loading Reagent (Fluidigm) per sample. The assay mix for the chip contained 0.7 µl primer pair mix (50 µM), 3.5 µl Assay Loading Reagent (Fluidigm) and 2.8 µl low TE buffer per primer pair. Chips were loaded with 5 µl sample mix and 5 µl assay mix. To measure gene expression, the chips were placed into the BioMark system (Fluidigm, South San Francisco, CA, United States) applying the "GE fast 96 × 96 PCR+Melt v2.pcl" protocol (Fluidigm). Each of the chips contained two technical replicates per sample and gene, two no-template controls (H_2O), one control for gDNA contamination (−RT) and one between plate control.

Juvenile Infection Experiment

Experimental Design and Treatment Groups

Within the first 24 h after birth, juveniles were removed from the adult tanks. Half of the brood was transferred to native salinity conditions and the other half to novel salinity conditions in a fully reciprocal design. Juveniles were fed twice a day with freshly hatched, nutrient enriched (Aqua Biotica orange+™) *Artemia salina* nauplii. Siblings were kept together in one non-aerated 1.5 l tank, of which one third of the water was exchanged daily. Once a day, left-over food was removed using single-use pipettes and mortality was documented.

Ten days post-hatch, juveniles received one of the four following *infection treatments*: (i) no injection – control (c), (ii) sham injection of autoclaved seawater (sw) with the equivalent salinity, i.e., 15 or 7 PSU, (iii) injection of *Vibrio alginolyticus* strain K01M1, which evolved for 90 days under laboratory condition at 15 PSU (v15) or (iv) *Vibrio alginolyticus* strain K01M1 that had evolved for 90 days under laboratory condition at 7 PSU (v7) (Goehlich, Roth et al., unpublished data). Pipefish were briefly taken out of the water and 2 µl of sterile seawater with or without bacteria was injected in the ventral part of the juveniles, using a Monoject™ insulin syringe (Coviden) with a sterile 30 Gauge needle. Subsequently, all juvenile siblings with the same treatment were placed in one 500 ml Kautex bottle containing seawater with the respective salinity of the 1.5 l tanks. Survival of juveniles was documented for 6 days and fish maintenance was according to the procedure described for 1.5 l tanks. One day post infection, one juvenile from each treatment (Kautex bottle) was euthanized and decapitated to assess expression of candidate genes. Standard body length was measured and whole-body samples were stored in RNA*later* overnight at 4°C and subsequently at −80°C.

Characterization and Evolution of *Vibrio alginolyticus* Strain Used for Injection

The *Vibrio alginolyticus* strain K01M1 used for injection of pipefish juveniles was isolated from a healthy pipefish caught in the Kiel Fjord (Roth et al., 2012a) and fully sequenced (Chibani et al., 2020b). The strain was evolved for 90 days either at 15 or 7 PSU [medium 101: 0.5% (w/v) peptone, 0.3% (w/v) meat extract, 1.5% (w/v) or 0.7% (w/v) NaCl in Milli-Q deionized water] (Goehlich, Roth et al., unpublished data). We used the same strain and evolved it at two different salinities to ensure that salinity is the only driver for potential differences in bacterial virulence, which could also be influenced by the presence of filamentous phages (Waldor and Mekalanos, 1996; Ilyina, 2015; Chibani et al., 2020a).

After 90 days the bacterial populations were diluted and plated onto *Vibrio* selective Thiosulfate-Citrate-Bile-Saccharose (TCBS) agar plates (Fluka Analytical^{TM}). The next day, single colonies from each plate were picked and grown overnight in medium 101 with the respective salinity. Subsequently, cultured bacteria were stored at −80°C as 33% glycerol stocks. For the infection experiment, part of the glycerol stocks were plated onto TCBS agar and one clone was grown in a 50 ml Falcon tube containing 30 ml medium 101 in the respective salinity for 24 h, at 25°C with shaking at 230 rpm. Overnight cultures were centrifuged for 20 min at 2,000 rpm. The supernatant was discarded and the cell pellet was resuspended in 3 ml sterile seawater (7 or 15 PSU, respectively) to achieve similar bacterial densities of 5×10^{10} ml^{-1}.

Juvenile Gene Expression

We measured gene expression of juveniles to assess the effect of (a) genetic adaptation (i.e., *origin salinity*) on gene expression, (b) *trans*-generational effects driven by an interaction between *acclimation salinity* and *developmental salinity* and (c) developmental plasticity induced by *developmental salinity*. Furthermore, we investigated (d) whether virulence differed in *V. alginolyticus* evolved at 15 or 7 PSU and whether juveniles from parents originating from a matching salinity were better adapted to *Vibrio* strains evolved at the respective salinity. Therefore, we selected genes from three functional categories, namely (i) immune response (ii), general metabolism, and (iii) gene regulation (DNA and histone modification) as described above for female pipefish *S. typhle* (section "Candidate Gene Expression of Females"). Compared to female gene expression, eleven genes from the categories (i–iii) were replaced by osmoregulation genes (iv). We selected osmoregulatory genes from teleost studies (**Supplementary Material 3**) and designed specific primers with Primer3Web (Koressaar and Remm, 2007; Untergasser et al., 2012; **Supplementary Material 4** and **Supplementary Table 4**). RNA extraction and quantification of gene expression were conducted as described in section "RNA Extraction and Reverse Transcription" with the following modifications due to a higher RNA yield: the fixed amount of RNA that was reverse transcribed into cDNA was 400 ng/sample (67 ng/μl) instead of 300 ng/sample (50 ng/μl) and pre-amplified cDNA was diluted 1:10 and instead of 1:20.

Statistics

All statistical analyses and visualizations were performed in the R 3.6.1 environment (RCoreTeam, 2020).

Life-History Traits

We used two-way ANOVAs to assess size and weight differences between males and females from different *origin salinity* as well as differences in brood size and in total length between juveniles at 10 days post-hatch. Fixed factors included *origin salinity* (salinity at samling sites of origin (two levels): *High* or *Low*), *acclimation salinity* (High or Low), *sex* of the pipefish (male or female) and the *sampling site* (Flens, Fehm, Falck, Salz, RuegN, or RuegS) nested in *origin salinity*. ANOVA of brood size additionally included the average body length of males exposed to a given treatment. Homogeneity of variances was tested by Fligner test and normal distribution of data by using the Cramer-von Mises normality test. The brood size was square root transformed to achieve normal distribution of residuals.

We performed two spearman-rank correlations using the function "ggscatter" (package: "ggpubr") to test for (1) correlation between the total length of adult pipefish and the salinity measured at the sampling site on the day of capture as well as (2) between brood size and average male size of sampling site. The brood size of males originating from high salinity and acclimated to low salinity conditions was removed from the correlation due to fungus infection. *Post hoc* tests were carried out using Tukey's "honest significant difference" (Tukey's HSD, package: "multcomp") (Hothorn et al., 2020).

Gene Expression of Parental Generation and Juveniles

From the Fluidigm output data, mean cycle time (Ct) and standard deviation (SD) for each of the two technical replicates were calculated. Expression measurements with a coefficient of variation (CV; CV = SD/Ct) larger than 4% were excluded from the study (Bookout and Mangelsdorf, 2003). For females, the combination of HDAC1 and HDAC3 were identified as the optimal reference genes [geNorm V < 0.15 (Vandesompele et al., 2002)] with high reference target stability (geNorm M ≤ 0.5), based on 155 samples (**Supplementary Table 5**) and 34 target genes (**Supplementary Table 2**) in *Qbase+3.0* (Hellemans et al., 2007).

In the analyses of juvenile gene expression one osmoregulation gene (15% NAs) and 36 samples were excluded from the study due to failed reactions on the Fluidigm chip in at least one of the duplicates. Samples with more than 10% excluded genes were omitted from the analysis, as many missing Ct values are indicative for insufficient sample quality. Remaining missing Ct values were substituted by the mean Ct for the given gene calculated from all other samples, as subsequent analyses are sensitive to missing data. Based on 559 samples and 47 target genes, the reference genes ASH and HDAC1 were selected using the same criteria as for pipefish females. From the geometrical mean of the two reference genes $-\Delta$Ct values were calculated to quantify relative gene expression.

Origin salinity (*High* or *Low*), *acclimation salinity* (High or Low) and *developmental salinity* (high or low) were defined as fixed factors, whereas *sampling site* (Flens, Fehm, Falck, Salz, RuegN, or RuegS) was nested within *origin salinity*. A Permutational Multivariate Analysis of Variance (PERMANOVA) was applied to gene expression (−ΔCt values) of all samples and target genes for each factor and every interaction of the fixed factors. The PERMANOVA [package: "vegan," function "adonis" in R (Oksanen et al., 2019)] was based on Euclidean distance matrixes with 1000 permutations (Beemelmanns and Roth, 2016b).

A post-hoc analysis of variance (ANOVA) for every gene was applied; though, to account for multiple testing, only factors and factor interactions identified as significant by the PERMANOVA were considered.

To visualize similarity/dissimilarity in gene expression among treatment groups, we performed PCAs [package: "ade4," function: "dudi.pca" and "s.class" (Dray and Dufour, 2007)]. To visualize significant differential gene expression among groups in heatmaps (package: "NMF," function: "aheatmap"), −ΔΔCt values for each gene were calculated as follows (Yuan et al., 2006):

$$-\Delta\Delta\mathrm{Ct} = \varnothing - \Delta\mathrm{Ct}\ \text{all samples} - \varnothing - \Delta\mathrm{Ct}\ \text{specific group}$$

Mortality of Juveniles Within the First 10 Days and Post-infection

Ten days post-hatch endpoint mortality of juveniles was analyzed as a ratio of "alive" vs. "dead" fish using a generalized linear model (package: "lme4", function: "glm") with binomial error and the following fixed factors: *Origin salinity* (*High* or *Low*), *acclimation salinity* (High or Low) and *developmental salinity* (high or low) and the *sampling site* nested in *origin salinity*. Significance was tested using ANOVA type two partial sums of squares, and models were simplified using Akaike information criterion (AIC) (Akaike, 1976). *Post hoc* tests were carried out using Tukey's honest significant difference (Tukey's HSD, package: "multcomp", function: "glht" (Hothorn et al., 2020)). Endpoint mortality of juveniles used in the infection experiment was analyzed as described above including *infection treatment* [control (c), sea water injection (sw), *Vibrio* 7 PSU (v7), and *Vibrio* 15 PSU (v15) injection] as an additional factor.

RESULTS

Pipefish Population Structure

Allele frequencies obtained at 11 microsatellite loci of 144 female pipefish sampled at six sampling sites along the German Baltic Sea coastline indicated gene flow or recently isolated populations with no or very little divergence on neutral genetic markers. The findings are based on a Bayesian clustering method using the software STRUCTURE (**Figure 2A**), global fixation index (F_{ST}) of 0.002 and pairwise F_{ST} (**Figure 2B** and **Supplementary Table 6**). Overall, the pairwise F_{ST} were low for all comparisons and with the exception of Falckenstein-Fehmarn (F_{ST} = 0.024) and Falckenstein-Flensburg (F_{ST} = 0.016) pairwise comparisons had a $F_{ST} \leq 0.01$ (**Supplementary Table 5**). Phylogenetic analyses suggest three branches in an unrooted tree with clusters comprised of pipefish from i) Ruegen North and Ruegen South, ii) Salzhaff and Flackenstein as well as iii) Flensburg and Fehmarn (**Figure 2C**).

Life-History Traits and Fungus Infection of Parental Generation

Pipefish Adults From Low Saline Environment Have a Smaller Body Size

Pipefish males and females caught in high *origin salinity* of the Baltic Sea were on average larger [mean ± SD, all high salinity sampling sites: 14.2 ± 2.1 cm; Flens: 14.4 ± 2.1 cm (n = 61), Falck: 14.5 ± 2.0 cm (n = 59), Fehm: 13.8 ± 2.1 cm (n = 59)] than those from the low *origin salinity* [all low salinity sampling sites: 12.8 ± 2.0 cm; Salz: 14.0 ± 2.1 cm (n = 53), RuegN: 11.7 ± 1.6 cm (n = 48), RuegS: 12.4 ± 1.5 cm (n = 52): **Figure 3A**]. We found an interaction in the total length of adult pipefish between *origin salinity* and *acclimation salinity* [ANOVA $F(1,320) = 7.4$, $p < 0.01$; **Supplementary Table 7A**] indicating that parental *acclimation salinity* negatively affected growth of adult pipefish depending on the *origin salinity*. Adults from high *origin salinity* tended to grow slower at low *acclimation salinity* compared to high *acclimation salinity* (Tukey's HSD, *H*H – *H*L: $p = 0.085$; **Supplementary Table 7B**), whereas *acclimation salinity* did not affect size of pipefish from low *origin salinity* (Tukey's HSD, *L*L – *L*H: $p = 0.535$: **Supplementary Table 7B**). Furthermore, all pairwise comparisons suggested that pipefish from high *origin salinity* were in general larger than pipefish from low *origin salinity* (Tukey's HSD, *L*L – *H*H: $p < 0.001$, *L*L – *H*H: $p < 0.001$, *L*H – *H*L: $p < 0.001$; **Supplementary Table 7B**). The significant factor sampling site, which was nested in *origin salinity* [ANOVA $F(4,320) = 11.2$, $p < 0.01$; **Supplementary Table 7A**] indicates that individuals from Salzhaff were larger compared to individuals from Ruegen North and Ruegen South but did not differ from pipefish caught at high *origin salinity* (Tukey's HSD, Salz – RuegN: $p < 0.001$; Salz – RuegS: $p < 0.001$; **Supplementary Table 7C**).

Pipefish from low origin salinity were smaller compared to pipefish from low *origin salinity* (correlation between salinity at sampling site and adult size, i.e., length (**Figure 3B**) and weight (**Supplementary Figure 7B**). Length and weight of animals were not corrected for age. When caught in spring, pipefish are usually all in the same age, as they were born the previous summer and reach sexual maturity around the catching date.

Pipefish From High Saline Environments Were More Susceptible to Fungus Infection When Exposed to Low Saline Conditions

Visible fungus infections of the brood pouch occurred in almost half of the pipefish males (47%) caught at high *origin salinity* and kept at low *acclimation salinity* (**Figure 4**). Fungus infections ranged from mild infections in the brood pouch not affecting brood size to a complete loss of the brood. Males from a high *origin salinity* that remained at high *acclimation salinity* as well as males from the low *origin salinity* had no symptoms of fungus infection regardless of the *acclimation salinity*.

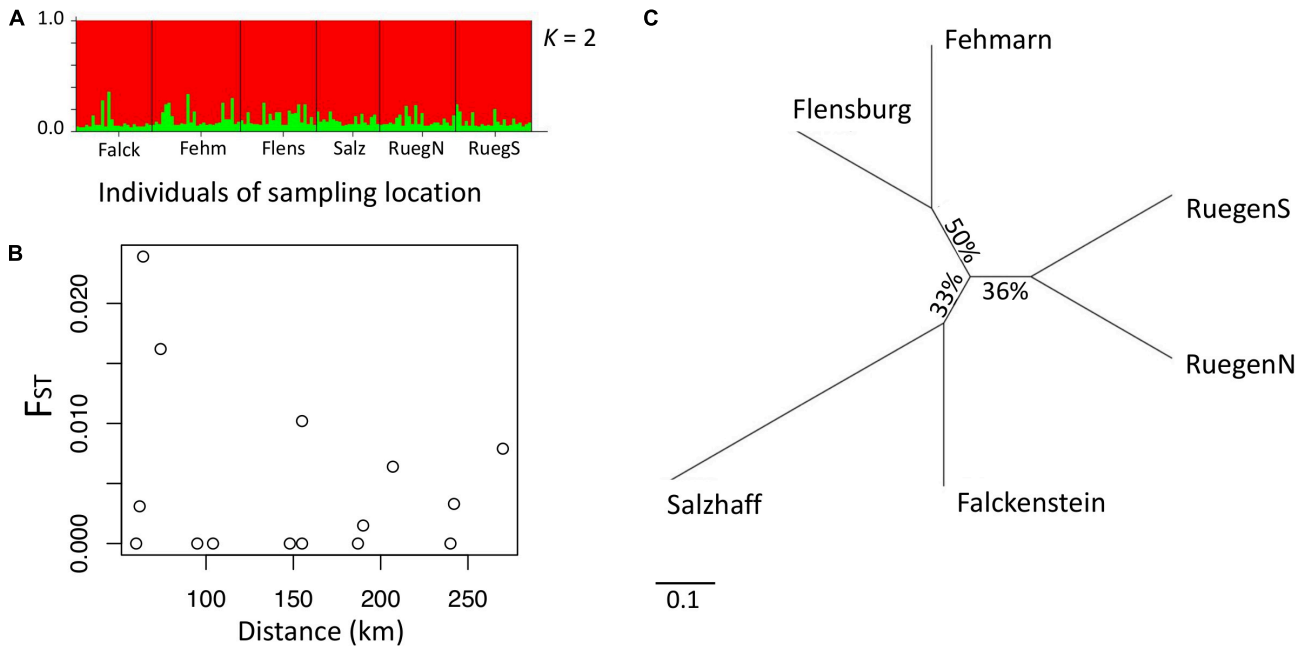

FIGURE 2 | Population structure of pipefish in the southern Baltic Sea**. (A)** STRUCTURE software results based on 11 microsatellite loci. Each individual is represented by a vertical line, which is colored according to the assigned groups (K = 2). **(B)** Plotting pairwise F_{ST} (y-axis) against distance between sampling sites (x-axis) does not reveal isolation by distance (waterway). **(C)** Unrooted phylogeographic PHYLIP tree. Distances indicate the relative divergence of microsatellite loci in pipefish between sampling sites. Scale bar represents nucleotide substitutions per site.

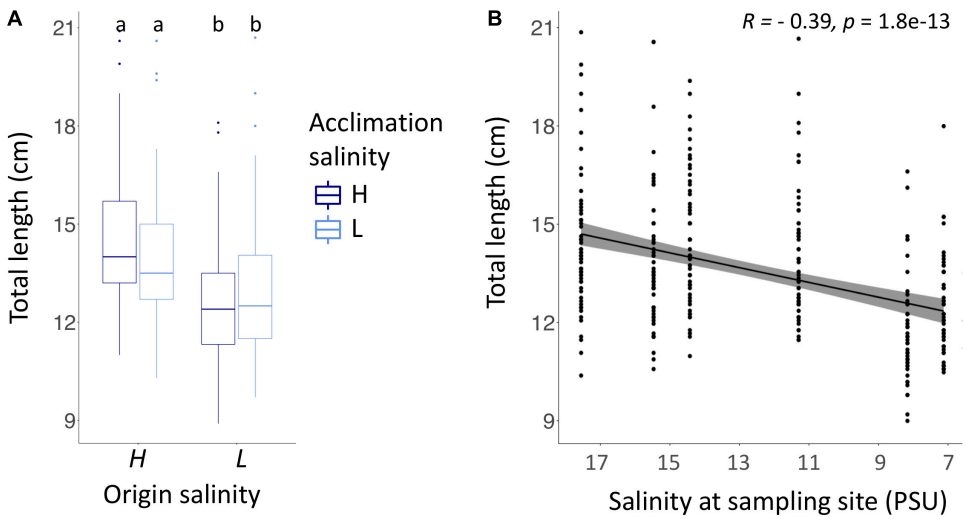

FIGURE 3 | Body length of adult pipefish **(A)** correlated with salinity at the sampling site **(B)**. **(A)** The total length of pipefish is shown for both *origin salinities* (high *(H)* – 15 PSU, low *(L)* – 7 PSU). Colors indicate whether the parental generation was exposed to a high (H, dark blue) or to low *acclimation salinity* (L, light blue). **(B)** The total length of pipefish after acclimation correlated with the salinity measured at the sampling site using spearman rank correlation. Gray bar indicates 95% confidence interval.

Brood Size

Males from a high *origin salinity* kept at high *acclimation salinity* had the largest brood size (*H*H, mean ± SD., 41.8 ± 23.4, n = 68, Tukey's TSD; **Supplementary Table 8B**) followed by males from low *origin salinity* kept at high salinity (*L*H, 27.8 ± 13.2, n = 58) or low salinity (LL, 25.2 ± 16.9, n = 58), which corresponds to the lower body size at low salinity (**Figure 3**). Pipefish from high *origin salinity* exposed to low *acclimation salinity* were frequently infected by a brood pouch fungus which reduced the brood size (*H*L 19.4 ± 15.2, n = 54; Tukey's TSD, *HL*-*HH*, $t = -4.8$, $p < 0.001$; **Supplementary Table 8B**). In contrast, *acclimation salinity* did not affect brood size of parents from low *origin salinity* (Tukey's TSD, *LL*-*LH*, $t = -0.4$, $p = 0.976$; **Supplementary Table 8B**). This divergent patterns

FIGURE 4 | Brood pouch fungus infections were present in 47% of fathers from high *origin salinity* kept at low *acclimation salinity*. Pipefish from low *origin salinity* independent of *acclimation salinity* and pipefish from high *origin salinity* kept at high *acclimation salinity* did not show any signs of fungus infection **(A)**. In males caught at high *origin salinity* and kept at low *acclimation salinity* fungi infections ranged from mild **(B)** to extreme **(C)** resulting in the complete loss of eggs and offspring.

caused an *origin salinity*:*acclimation salinity* interaction [ANOVA $F(1,109) = 9.0$, $p = 0.003$; **Supplementary Table 8A**]. Larger brood size was in general driven by a larger total body length of male pipefish (Spearman rank correlation, $R = 0.43$, $p < 0.001$, $n = 234$; **Figure 5**).

Two Immune Genes Are Upregulated in Females From a High Origin Salinity

Origin salinity had an impact on the expression of immune genes in female pipefish (PERMANOVA, *immune* $F(1,146) = 3.1$, $p = 0.010$, $n = 155$, **Supplementary Table 9**, **Supplementary Figure 9A**), in particular of the innate immune system [PERMANOVA, *innate* $F(1,146) = 4.0$, $p = 0.002$; **Supplementary Table 9**]. *Lectin protein type I* (*Lecpt1*), a pathogen recognition receptor, and *chemokine 7* (*ck7*), a gene encoding a protein responsible for chemotaxis in blood cells, were upregulated in pipefish from low *origin salinity* in contrast to high *origin salinity* females. Low *acclimation salinity* caused a slight upregulation in the expression of histone modification gene *histone deacetylase 6-like* (*hdac6*) [PERMANOVA, *silencing* $F(1,146) = 2.9$, $p = 0.044$; **Supplementary Table 9**, **Supplementary Figure 9A**].

Life-History Traits, Survival and Gene Expression of Offspring Generation

Juveniles From Low Origin Salinity Parents Have Higher Survival Rates and Are Smaller

In the first 10 days after hatching, juvenile survival was impacted by *origin salinity* and *acclimation salinity* of the parental generation *origin salinity*:*acclimation salinity* interaction, GLM, $\chi^2_1 = 6.1$ ($p = 0.013$, $n = 235$; **Supplementary Table 10A**; **Figure 6**). There was no difference in survival of juveniles from parents that were continuously exposed to the same salinity (i.e., matching *origin* and *acclimation salinity*) (Tukey's HSD, *LL* – *HH*: $z = -3.4$, $p = 0.783$; **Supplementary Table 10B**). Similarly, juvenile survival did not differ for non-matching *origin salinity* and *acclimation salinity* *LL* – *LH*: $z = -4.7$, $p = 0.842$; *HH* – *HL*: $z = -2.1$, $p = 0.148$; **Supplementary Table 10B**). However, juveniles from high *origin salinity* parents exposed to high *acclimation salinity* in the lab (*LL*) had higher survival rates compared to juveniles from high *origin salinity* exposed to low *acclimation salinity* (Tukey's HSD, *LL* – *HL*: $z = -3.4$, $p = 0.043$; **Supplementary Table 10B**). The *origin salinity*:*sampling site* effect suggest that patterns at single sampling sites differ. In particular, Flensburg (*origin salinity*: 17 PSU "high") offspring exposed to high *developmental salinity* had reduced survival rates, when parents were acclimated to low instead of high salinity (Tukey's HSD; Hh – Lh: $z = -4.4$, $p = 0.046$; **Supplementary Table 10B**). Following the same pattern of non-matching *acclimation salinity*, Ruegen South (*origin salinity*: 7 PSU "low") offspring exposed to low *developmental salinity* had reduced survival, when parents were kept at high *acclimation salinity* (Tukey's HSD; Hl – Ll: $z = 5.8$, $p < 0.001$; **Supplementary Table 10B**). This suggests that exposure of parents to novel salinities can negatively impact juvenile survival when juveniles experience salinity conditions, which did not match parental *acclimation salinity*.

Overall higher survival at high *developmental salinity* compared to low *developmental salinity* (*developmental salinity*, GLM, $\chi^2_1 = 192.8$, $p = 0.031$; **Figure 6**; **Supplementary Table 10A**) indicates that low salinity imposes stress on pipefish juveniles regardless of the *origin salinity*. An exception are juveniles from Ruegen North (*sampling site*, GLM, $\chi^2_4 = 24.1$, $p < 0.001$; **Supplementary Figure 9A**) where juvenile survival was not affected by *developmental salinity* (GLM, $\chi^2_1 = 0.1$, $p = 0.766$).

Ten days after hatching, juveniles from high *origin salinity* were larger (3.18 cm ± 0.37, $n = 408$; **Figure 6**) than juveniles from low *origin salinity* sampling sites (2.95 cm ± 0.37, $n = 405$) [*origin salinity*, ANOVA $F(1,782) = 86.2$, $p < 0.001$; **Supplementary Table 11**; **Figure 6**]. While *acclimation salinity*, i.e., mating and male pregnancy, had no effect on size of juveniles [*acclimation salinity*, ANOVA $F(1,782) = 2.2$, $p < 0.136$; **Supplementary Table 11**], low *developmental salinity* reduced offspring size [*developmental salinity*, ANOVA $F(1,782) = 17.4$, $p < 0.001$; **Supplementary Table 11**] suggesting that low salinity levels are stressful for pipefish offspring and reduce their growth rates.

Juvenile Survival Is Reduced After Injections and at Low Salinity

Ten days post hatch, juvenile pipefish were challenged either with *Vibrio alginolyticus* bacteria evolved at 15 PSU, 7 PSU, autoclaved seawater (sham injection) or not treated at all (control) and survival was measured 6 days post infection, i.e., approximately 16 days post hatch. Non-challenged control groups had the highest survival rates (Mean ± SD.; 83.0% ± 32.2, $n = 237$; **Figure 7**). The injection itself decreased survival of juveniles by at least 10% in all salinity treatments combined, regardless whether seawater (66.9% ± 38.2, $n = 192$), *Vibrio* evolved at 15 PSU (73.0% ± 36.8, $n = 190$) or 7 PSU (66.7% ± 38.6, $n = 192$; **Figure 7**) was administered. *Vibrio* strains evolved at 15 PSU caused a higher mortality in juveniles from high

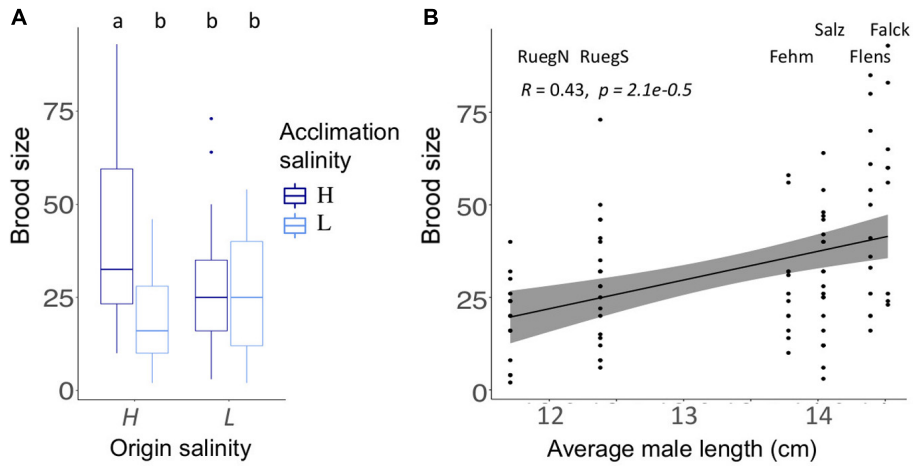

FIGURE 5 | Parental life-history traits. **(A)** Number of juveniles (brood size) is shown for pipefish with high (*H*) or low (*L*) *origin salinity*. Color indicates *acclimation salinity* in the lab (H: 15 PSU – dark blue and L: 7 PSU – light blue). Different letters indicate significant differences (Tukey's HSD, $p < 0.05$). **(B)** Brood size correlated positively with the average size of male pipefish from each sampling site. The *sampling sites* are presented above the data points. The gray bar along the regression line indicates the 95% confidence interval. A more detailed figure visualizing the differences between the single origins can be found in the **Supplementary Table 7C**.

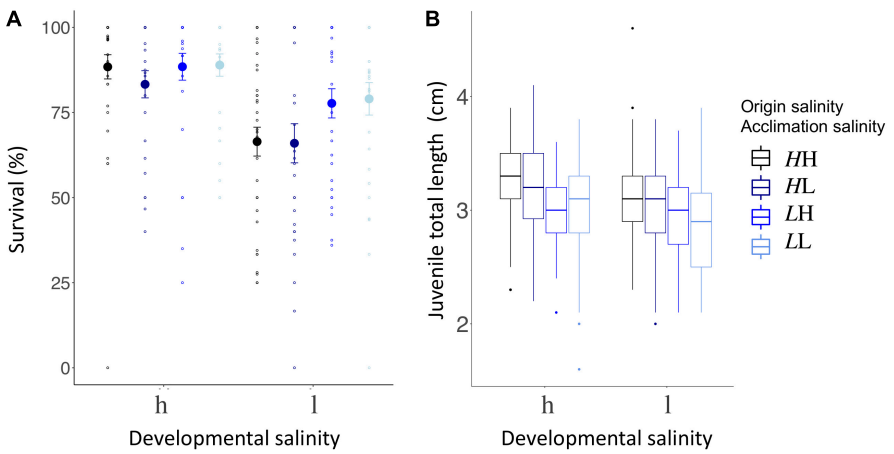

FIGURE 6 | Juvenile life history parameters **(A)** survival rate (%), **(B)** body length (cm). **(A)** The percentage of juveniles surviving the first 10 days post-hatch (%) are plotted on the *y*-axis. The *x*-axis indicates the *developmental salinity* after hatching (h – high/15 PSU, l – low/7 PSU). Italic letters and colors specify the *origin salinity* of the parental generation (*H*: 15 PSU, black and dark blue; *L*: 7 PSU blue and light blue). The 2nd letter indicates the *acclimation salinity* in the lab (H: 15 PSU, black and blue; L: 7 PSU, dark and light blue). **(B)** Juvenile size (cm) 10 days post hatch is plotted on the *y*-axis. Labeling and color code correspond to panel **(A)**.

origin salinity regardless of *acclimation salinity* compared to juveniles from low *origin salinity* with low parental *acclimation salinity* (*origin salinity* × *acclimation salinity* × *treatment,* GLM, $\chi^2_1 = 13.0$, $p = 0.005$; **Supplementary Table 12A**; Tukey's HSD; *L*Lv15 – *H*Hv15: $z = -3.4$, $p = 0.046$; *L*Lv15 – *H*Lv15: $z = -3.5$, $p = 0.038$; **Supplementary Table 12B**). When fathers from low *origin salinity* were exposed to high *acclimation salinity* these positive effects on offspring survival were lost (Tukey's HSD, *L*Hv15 – *H*Hv15 $z = -1.5$, $p = 0.971$; **Supplementary Table 12B**). This suggests that mis-matching salinity levels between the parental and juvenile generation can lead to reduced survival rates.

Juvenile survival was in general higher in high *developmental salinity* conditions (71.1% ± 36.7, $n = 421$) compared to low *developmental salinity* conditions (58.6% ± 36.7, $n = 392$) (GLM, *developmental salinity,* $\chi^2_1 = 40.2$, $p = 0.001$; **Supplementary Table 12A**) suggesting that low salinity levels are a stressful environment for pipefish development. An adaptation to low salinity may result in an increased fitness as juveniles from low *origin salinity* fathers (69.8% ± 40.1, $n = 405$) had in general a higher survival rate compared to juveniles from high *origin salinity* fathers (60.6% ± 37.7, $n = 408$; GLM, *origin salinity,* $\chi^2_1 = 9.1$, $p = 0.003$, **Supplementary Table 12A**).

An effect of *sampling site* nested in *origin salinity* (GLM, $\chi^2_4 = 39.5$, $p < 0.001$; **Supplementary Table 12A**) indicates that survival patterns for each sampling site within the *origin salinity* categories are diverse. The statistical diversity may be a result of the high variation in survival rates within a single

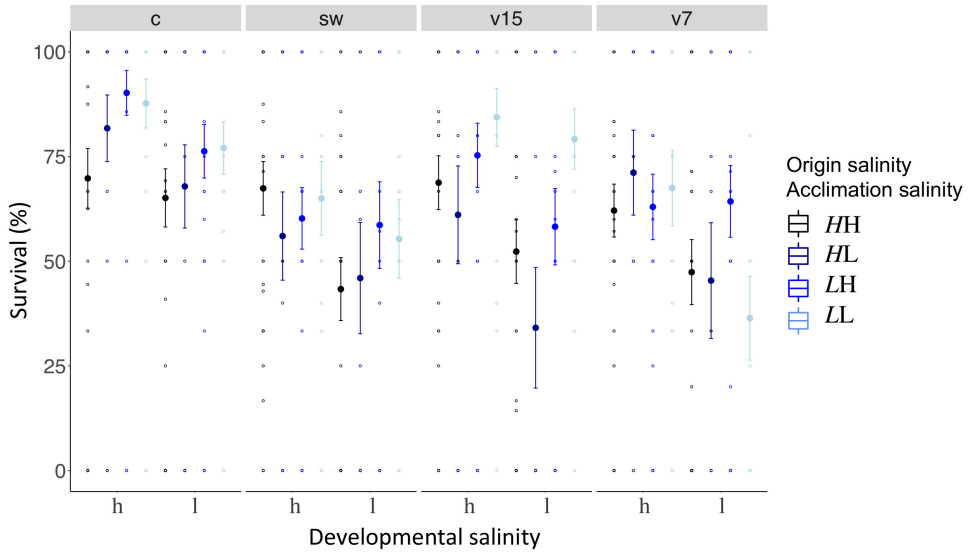

FIGURE 7 | Juvenile survival 6 days post infection. Juvenile survival 6 days post infection is plotted on the *y*-axis. The *x*-axis indicates the *developmental salinity* (h – high/15 PSU, l – low/7PSU). Italic letters and colors specify the *origin salinity* of the parental generation (*H*: 15 PSU, black and dark blue; *L*: 7 PSU, blue and light blue). The 2nd letter and colors indicate the *acclimation salinity* in the lab (H: 15 PSU, black and blue; L: 7 PSU, dark and light blue). Each *treatment* is represented by one panel, i.e., control (c) or injection with seawater (sw), *Vibrio* strain evolved at 15 PSU (v15), or at 7 PSU (v7).

treatment, which sometimes ranged from 0 to 100%. Combining the survival rates of all three sample sites of one *origin salinity* resulted in more robust and conclusive results as described in the first paragraph of this section.

Matching Parental Acclimation and Juvenile Developmental Salinity Results in Similar Juvenile Gene Expression Patterns of Adaptive Immune Genes

An *origin salinity* effect indicates that gene expression of juveniles differs depending on the salinity they originated from [PERMANOVA, *all genes*; *origin salinity* $F(1,523) = 4.2$, $p = 0.003$, $n = 559$; **Figure 8**, **Table 2**]. Such signs of genetic adaptation were found in genes associated with the innate immune system [PERMANOVA, *innate*, $F(1,523) = 9.2$, $p = 0.001$] and with osmoregulation [PERMANOVA, *osmo*, $F(1,523) = 4.1$, $p = 0.003$; **Figure 8**, **Table 2**]. Single ANOVAs suggested that this effect was driven by five genes. Whereas the expression of the pathogen reception recognition gene *lectin protein type II (lectpt2)* was induced in juveniles from low *origin salinity* parents, the expression of the following genes was upregulated in juveniles from high *origin salinity* parents: *immunoglobulin light chain* (*IgM*; pathogen recognition), *heat shock protein 70 kDa* (*hsp70*; osmotic stress response); *voltage gated potassium channel* (*kcnh8*; cell volume regulation), *prolactin* (*prl*; ion uptake promotion and ion secretion inhibition). The genetically induced upregulation of osmoregulatory genes suggests an adaptation to low salinity levels.

Juvenile gene expression did not provide further evidence for genetic adaptation, tested as an interaction between *origin salinity* and *acclimation salinity* [PERMANOVA, *all genes* $F(1,523) = 0.5$, $P = 0.858$]. *Trans*-generational plasticity could also not be detected in the interaction between *acclimation salinity* and *developmental salinity* [PERMANOVA, *all genes* $F(1,523) = 0.6$, $P = 0.730$].

Exposing parents to low *acclimation salinity* led to an expression induction of two genes in juveniles [PERMANOVA, *complement*; *acclimation salinity* $F(1,523) = 4.6$, $p = 0.014$]. Both genes are associated with the complement system: *Complement component 3* (*c3*, complement system activation) and *Complement component 9* (*c9*, membrane attack complex). In addition to this parental effect a *trans*-generational effect was observed as a *parental acclimation salinity*: *developmental salinity* interaction effect on adaptive immune gene expression [PERMANOVA, *adaptive* $F(1,523) = 2.6$, $p = 0.034$; **Figure 8B**, **Table 2**]. Gene expression was lower in four out of seven adaptive immune genes when *acclimation salinity* and *developmental salinity* were matching compared to non-matching conditions (**Supplementary Figure 13**): *Human immunodeficiency virus type l enhancer 2* (*hivep2*, transcription factor, MHC enhancer binding) and *3* (*hivep3*; transcription factor, MHC enhancer binding), *B-cell receptor-associated protein* (*becell.rap31*, T- and B-cell regulation activity) and *immunoglobulin light chain* (*igM*; antigen/pathogen recognition). The reduction in gene expression of immune genes can hint at a reduced stress level in offspring when parents were acclimated to the same salinity as their offspring.

Developmental plasticity allows juveniles to quickly respond to present salinity levels. Low *developmental salinity* resulted in higher expression of six genes. *Complement component 3 (c3)* is involved in the complement system [PERMANOVA, *comple*; *developmental salinity* $F(1,523) = 4.6$, $p = 0.014$; **Figure 8**, **Table 2**], *prolactin* (*prl*, ion uptake promotion and ion secretion inhibition) is associated with osmoregulation [PERMANOVA,

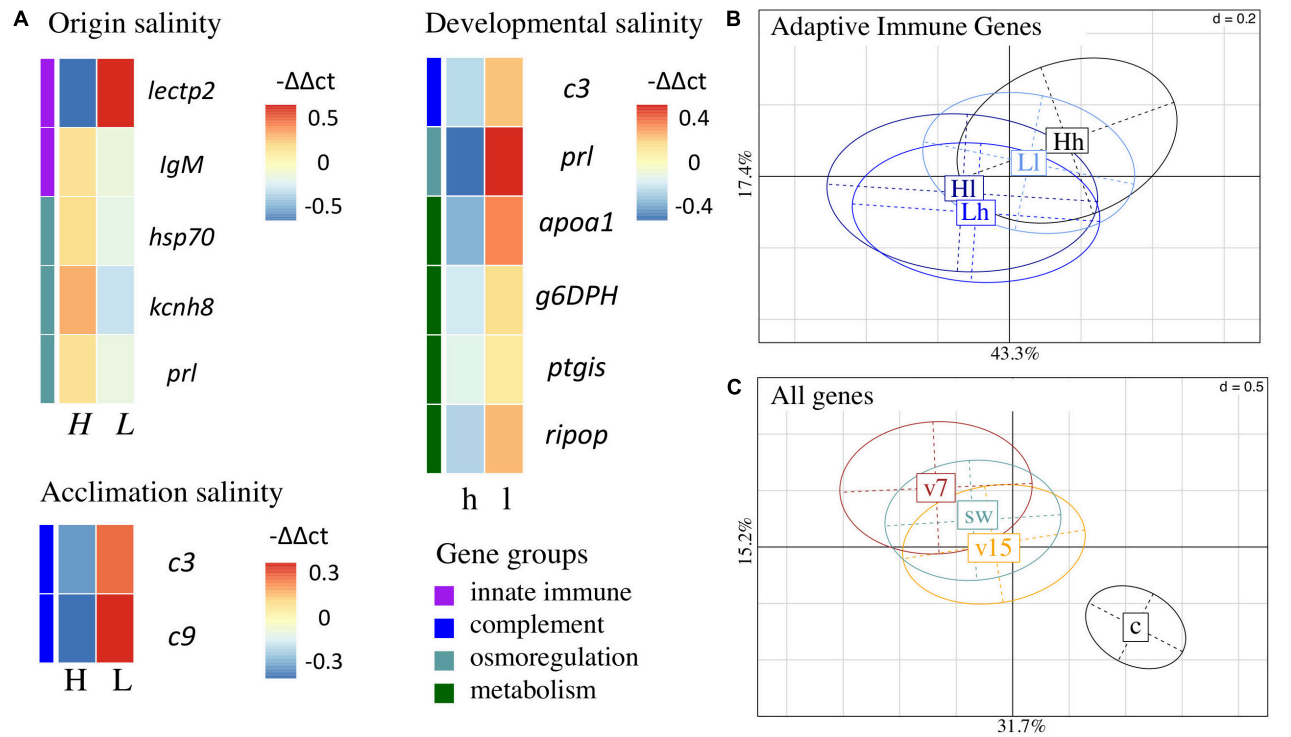

FIGURE 8 | Gene expression patterns of juveniles. **(A)** Non-hierarchical gene expression heatmap for genes showing differential expression (-ΔΔCt) in response to *origin salinity*, *acclimation salinity* or *developmental salinity*. Genes are sorted by gene groups which are assigned with different colors (purple: innate immune system, blue: complement system, turquoise: osmoregulation, green: metabolism). **(B)** Principal component analysis (PC1: 43.3%; PC2: 17.4%) of adaptive immune genes for significant interaction between parental *acclimation salinity* [H – high (15 PSU), L – low (7 PSU)] and *developmental salinity* (h, l). Four out of seven adaptive immune gene were upregulated (**Supplementary Figure 13**), when parental *acclimation salinity* and juvenile *developmental salinity* did not match, i.e., Hl and Lh, compared to a matching *acclimation* and *developmental salinity*, i.e., Hh and Ll [PERMANOVA, *adaptive* $F(1,523) = 2.6$, $p = 0.034$]. The confidence ellipse explains 20% of the variability. **(C)** Principal component analysis (PC1: 31.7%; PC2: 15.2%) of gene expression patterns caused by juvenile *treatment*, i.e., injection with *Vibrio alginolyticus* evolved at 15 PSU (v15, orange), *V. alginolyticus* evolved at 7 PSU (v7, brown), sham injection with seawater (sw, blue, green) or untreated control (c, black). The confidence ellipse explains 20% of the variability.

osmo; *developmental salinity* $F(1,523) = 3.3$, $p = 0.028$] and *apolipoprotein A1* (*apoa1*, antimicrobial activity), *glucose 6 phosphate dehydrogenase* (*g6DPH*, pentose phosphate pathway), *prostaglandin I2 Synthase* (*ptgis*, Lipid and fatty acid metabolism), *ribosomal protein* (*ripop*, Translation process) are related to the metabolism [PERMANOVA, *meta*; *developmental salinity* $F(1,523) = 4.6$, $p = 0.014$].

Finally, we wanted to test whether genetic background, i.e., *origin salinity*, *acclimation salinity* of the parents and the *developmental salinity* influenced the ability of juveniles to cope with infections of the opportunistic pathogen *V. alginolyticus*, which evolved in the lab at either 15 or 7 PSU. However, we found no interaction between any salinity regime of parents or juveniles interacting with gene expression after juvenile infection. An injection, regardless of the component, i.e., autoclaved seawater, *V. alginolyticus* evolved at 15 PSU or 7 PSU caused similar changes in gene expression patterns that could only be differentiated from the untreated control group. In 24 genes injections induced gene expression [PERMANOVA, *all genes*; $F(3,523) = 11.3$, $p = 0.001$; **Supplementary Figure 14**], including genes from all groups. In five genes, injections reduced gene expression compare to the control group. Using *post hoc* tests on ANOVAs of single genes, we found no differences in gene expression between the three injection treatments.

DISCUSSION

We have investigated the role of genetic adaptation and phenotypic plasticity as well as their interaction on the ability of the broad-nosed pipefish *Syngnathus typhle* to cope with changes in salinity levels. *S. typhle* is a marine teleost, which originally invaded from the North Sea into the Baltic Sea after its formation about 8000 years ago (Wilson and Veraguth, 2010). The brackish salinity environment in the Baltic Sea imposes osmoregulatory stress on marine animals and is thus assumed to be an important driver for genetic divergence and adaptation to local condition (Berg et al., 2015; Guo et al., 2015; Johannesson et al., 2020). Strong genetic clines over the Baltic Sea salinity gradient were shown for a diversity of species (Johannesson et al., 2020) with strong patterns of local salinity adaptation (Leder et al., 2021). Here, we focused on six pipefish populations from the German coastline

TABLE 2 | PERMANOVA results of juvenile gene expression.

		All genes		Immune		Innate		Adaptive		Complement		Metabolism		Osmoregulation		Epigenetics	
	df	F Model	Pf(> F)	F Model	Pf(> F)	F Model	Pf(> F)	F Model	Pf(> F)	F Model	Pf(> F)	F Model	Pf(> F)	F Model	Pf(> F)	F Model	Pf(> F)
Origin salinity (Origin sal)	1	**4.2**	**0.003***	5.2	**0.001***	**9.2**	**0.001***	1.4	0.21	2.3	0.098	1.5	0.203	**4.1**	**0.003***	0.7	0.508
Acclimation salinity Acclim sal)	1	1.5	0.15	1.6	0.129	1.2	0.277	0.5	0.715	**4.6**	**0.014***	1.1	0.326	1	0.411	2.6	0.578
Developmental salinity (Devo sal)	1	**3.2**	**0.005***	**2.5**	**0.034***	1.7	0.136	1.3	0.234	**3.3**	**0.028***	**4.2**	**0.020***	**6.4**	**0.001***	1.2	0.298
Treatment (Treat)	3	**11.3**	**0.001***	**13.9**	**0.001**	**17.5**	**0.001***	**16.8**	**0.001***	**6.3**	**0.001***	**2.5**	**0.025***	**9.1**	**0.001***	**3.2**	**0.005***
Origin sal:Acclim sal	1	0.5	0.858	0.4	0.93	0.3	0.91	0.7	0.582	0.6	0.535	0.2	0.908	0.8	0.525	1.6	0.182
Origin sal:Devo sal	1	0.6	0.73	0.6	0.783	0.6	0.663	0.6	0.642	0.3	0.74	0.3	0.798	1.2	0.265	0.5	0.672
Acclim sal:Devo sal	1	1.6	0.141	1.8	0.088	2.1	0.639	**2.6**	**0.034***	1.3	0.26	0.8	0.436	1.2	0.311	2.2	0.093
Origin sal:Treat	3	1.1	0.289	1.2	0.255	0.9	0.502	0.6	0.782	1.5	0.19	2.1	**0.037***	0.7	0.796	0.6	0.762
Acclim sal:Treat	3	0.6	0.933	0.4	0.995	0.3	0.999	0.8	0.633	0.3	0.947	0.9	0.503	0.9	0.526	0.5	0.881
Devo sal:Treat	3	0.8	0.675	0.9	0.603	0.8	0.671	1.1	0.336	0.6	0.722	0.9	0.453	0.8	0.678	0.6	0.815
Origin sal:sampling site	4	**2.9**	**0.001***	**3.1**	**0.001***	**3.5**	**0.001***	**3.2**	**0.001***	**2.2**	**0.036***	**2.3**	**0.023***	**2.1**	**0.005***	**3.7**	**0.003***
Origin sal:Acclim sal:Devo sal	1	1.2	0.251	1	0.372	1.3	0.221	1.1	0.354	0.3	0.809	1.4	0.214	0.7	0.633	2.2	0.089
Origin sal:Acclim sal:Treat	3	0.8	0.725	0.8	0.705	0.7	0.805	0.9	0.513	1	0.42	0.9	0.498	0.8	0.669	0.9	0.482
Origin sal:Devo sal:Treat	3	0.9	0.604	0.6	0.877	0.5	0.917	0.4	0.953	0.6	0.72	1.2	0.28	1.1	0.307	0.9	0.465
Acclim sal:Devo sal:Treat	3	0.9	0.534	0.9	0.582	0.8	0.618	1.3	0.202	1.1	0.37	0.7	0.628	0.8	0.665	0.9	0.541
Origin sal:Acclim sal:Devo sal: Treat	3	0.6	0.933	0.5	0.983	0.5	0.956	0.5	0.912	0.4	0.918	0.6	0.718	0.9	0.507	0.7	0.664
Residuals	523																
Total	558																

*A PERMANOVA was applied to gene expression (−ΔCt values) of all 558 samples for all genes (47), including immune genes comprised of the innate, adaptive and complement genes, as well as genes involved in metabolism, osmoregulation and epigenetics, e.g., methylation or histone modification. Results are based on Euclidian distance matrices with 1,000 permutations. Significant p-values in bold and marked with *.*

of the Baltic Sea, out of which three originated from a relatively high saline environment (14–17 PSU), and three from a relatively low saline environment (7–11 PSU). By taking the two salinity regimes into account, our experiment permitted to test both for local adaptation and for phenotypic plastic acclimation to different salinities. Pipefish from all sampling sites displayed low levels of neutral differentiation. This provides good opportunities for studying local adaptation of phenotypically differentiated populations because observed phenotypic differences are likely to reflect genes under selection rather than genetic differences resulting from stochastic processes such as drift (Sunde et al., 2020).

Syngnathus typhle caught in the Baltic Sea high salinity environments (14–17 PSU) were smaller (mean size in this study: 14.2 ± 2.1 cm) than those populating the marine realm with more than 28 PSU (mean size animals caught between 28 and 36 PSU: 18.7 cm (Rispoli and Wilson, 2008) or 15.5 cm (Gurkan and Taskavak, 2007), but larger than those sampled in Baltic Sea low salinity environments, i.e., mean size in this study: 12.8 cm ± 2.0 cm, pipefish sampled at 5.5 PSU around Askö (Sweden): 14.5 cm (Rispoli and Wilson, 2008) or at 7 PSU around Gotland: 14.0 ± 1.1 cm (females), 13.4 ± 1.1 cm (males) (Berglund et al., 2017). This suggests that osmoregulation is costly (Rolfe and Brown, 1997; Boeuf and Payan, 2001) and that the negative impact of low salinity can potentially not be fully compensated through local adaptation. As such, trade-offs for osmoregulation may reduce growth rates, which ultimately result in a decreased fitness. Studies of other marine teleosts that originated from fully marine environments, e.g., sticklebacks and cod, suggested that high growth rates at intermediate salinity levels (10–20 PSU) are possible, especially when the salinity levels reclose or slightly above isosmotic levels (Dutil et al., 1997; Imsland et al., 2001; Heckwolf et al., 2018).

In this previous study (Nygård et al., 2019), the body weight or length of the offspring correlated with the parental size. Larger females produced larger eggs, and fathers with larger body size gave birth to larger juveniles (Nygård et al., 2019). To this end, both the parental body size and a resource-allocation trade off imposed by an increased energy demand for osmoregulation can explain the reduced embryonal growth in the low saline environment (Boeuf and Payan, 2001). In the here presented survival experiment, juveniles from low origin salinity parents, i.e., RuegS, RuegN and Salzhaff, survived better compared to high origin salinity parents, i.e., Flensburg, Falckenstein, Fehmarn. Their induced survival was neither influenced by the parental acclimation salinity nor by the developmental salinity or the exposure to *Vibrio* bacteria. We thus suggest two alternative parental care strategies: (i) large broad-nosed pipefish parents can invest in larger brood and offspring size as suggested for the larger animals originating from high saline populations that produced more offspring of a larger size, while (ii) small parents may rather invest in offspring survival (Nygård et al., 2019) via genetically determined gene expression patterns as suggested by the induced survival of offspring from the generally smaller parents originating from low saline environments.

Such genetically determined gene expression patterns that are inherited from generation to generation can be indicative signs for local adaptation (Larsen et al., 2011; Fraser, 2013; Heckwolf et al., 2020). In this study, juveniles from animals caught in high saline environments generally had an induced expression of osmoregulation genes. We suggest that the here observed genetically determined level of osmoregulation gene expression in pipefish is an adaptation to local salinity levels. The upregulation of the immune gene *lecpt2* in the juveniles from low salinity parents may be an indicator for an adaptation towards the specific local pathogen community. Adaptive gene expression levels in the immune system can result in a faster and stronger and eventually more effective immune responses (Swaggerty et al., 2008). In contrast, pipefish from low origin salinity may rather suffer stress induced by the above stated resource allocation trade-off, which decreases the resources available for the innate immune system.

Under stress, animals are more susceptible to infections with pathogens, which may turn opportunistic pathogens into causative agents of deadly diseases (Boyett et al., 2007; Poirier et al., 2017; Sullivan and Neigel, 2018). Furthermore, low saline environments have been suggested to select for increased pathogenic virulence, e.g., due to changes in gene expression (Hase and Barquera, 2001) and biofilm formation (Dayma et al., 2015). This is in line with the observed brood pouch infections during pregnancy that massively impacted fathers adapted to a high origin salinity but exposed to low acclimation salinity. The number of offspring was reduced and the offspring had a smaller body size. Fathers caught at low origin salinity (both *LL*: low origin salinity, low acclimation salinity and *LH*: low origin salinity, high acclimation salinity) did not show signs of brood pouch infection, which gives support for our hypothesis that these animals were locally adapt to low saline environments and the associated pathogens. Juveniles exposed to *Vibrio* bacteria survived better when their parents originated from low saline waters. This suggests that in low saline waters selection induced by the potentially more virulent and more prevalent microbial infectious agents must have resulted in pipefish that are less susceptible (i.e., either more resistant or more tolerant) to infections with opportunistic *Vibrio* bacteria.

Juveniles are expected to have advantages when exposed to the same environment as their parents (Sunday et al., 2014; Roth et al., 2018). An interaction of the parental acclimation salinity and the juvenile developmental salinity is generally interpreted as an indicator for *trans*-generational plasticity (Uller et al., 2013; Heckwolf et al., 2018). In contrast to previous experiments focusing on *trans*-generational plasticity and immune priming in pipefish (Beemelmanns and Roth, 2016b; Beemelmanns and Roth, 2017; Roth and Landis, 2017), the adaptive *trans*-generational plastic effects identified in this study were limited. Even though survival of juveniles was higher in matching parental acclimation and developmental salinity, the effect was driven by the genetic adaptation and not the parental acclimation, as indicated by the strong origin salinity effect. The same applied to juvenile growth, which was imposed both by origin salinity and by the developmental salinity, but not by acclimation salinity. However, parental acclimation shifted expression of genes involved in complement and adaptive immune systems. As such, parental acclimation to low salinity (main effect)

induced the expression of genes of the complement system. Non-matching parental acclimation and developmental salinity (interaction) upregulated genes of the adaptive immune system compared to matching parental acclimation and developmental salinity. In contrast to the above discussed upregulation of innate immune genes, an upregulation of the complement and adaptive immune system is indicative for a clear response toward prevailing parasites and pathogens, due to the specificity of the adaptive immune system (Janeway, 2005). The complement system links the innate to the specific adaptive immune system. Their joint induction could give evidence for a shift in the microbial pathogen community in non-matching environments to which the specific arm of the immune system has to react. However, final support would enquire the genotyping of the microbial pipefish gut community.

The limited presence of *trans*-generational plasticity gives only marginal support for our hypotheses and is in strong contrast to previous experiments performed with the same model system, where the genetic background was mostly ignored and experiments focused only on one population [Roth et al., 2012b; Beemelmanns and Roth, 2016b (Gotland, Sweden); Beemelmanns and Roth, 2017; Wackerballig (Gelting, Germany), Roth and Landis, 2017; Wackerballig (Gelting, Germany)]. The here performed experiment allows us to at least partially disentangle genetic adaptation and *trans*-generational plasticity and suggests that selection and the resulting genetic adaptation is a lot stronger than the impact of *trans*-generational plasticity. To this end, the unexpected limited identification of *trans*-generational plastic effects could indicate that we are generally overestimating *trans*-generational plasticity in experiments that ignore genetic background, as genetic adaptation is intermingled with the phenotypic plastic components. Alternatively, we have potentially not identified all present signs of *trans*-generational plasticity in this experiment as the populations are too distinct due to their history of genetic adaptation hindering the identification of *trans*-generational plastic effects. By taking the genetic adaptation into account, we suggest that the probability to identify existing phenotypic plastic effects is lower, as the impact of phenotypic plastic effects is weaker than the impact of genetic differences among populations.

Populations that migrate into a new habitat are under strong selection for genetic adaptation toward the novel environmental condition. They go through a bottleneck, which results in populations that are diverged from their ancestral populations (Johannesson et al., 2020) and are characterized by a reduced genetic diversity (Johannesson and Andre, 2006). In another study this reduced genetic diversity as a consequence of genetic adaptation negatively impacted the individual phenotypic plasticity of sticklebacks populating low salinity regions of the Baltic Sea (DeFaveri and Merila, 2014; Hasan et al., 2017). In a stable salinity environment, we would thus expect that genetic adaptation had resulted in reduced phenotypic plasticity and lower performance in the ancestral environment (DeWitt et al., 1998; Schneider and Meyer, 2017). In contrast to our expectation, juvenile survival of parents from low salinity origins was not reduced at high developmental salinity suggesting that genetic adaptation toward low salinity conditions did not result in a reduction of phenotypic plasticity. Along the same line, the smaller size of juveniles from parents originating from low salinity environments is no indicator for reduced plasticity either. The smaller phenotype (at the same age) was more likely a result of the reduced parental size (Nygård et al., 2019) which can be an adaptation to low salinities (McGuigan et al., 2011). The strong horizontal and vertical salinity gradients, the variations in precipitation and erratic saltwater inflow from the North Sea are causing strong salinity fluctuations in the coastal environments across the Baltic Sea (Bock and Lieberum, 2017) most likely selecting against the loss of phenotypic plasticity.

The isosmotic level of many marine fish is equivalent to around 12 PSU (Schaarschmidt et al., 1999) or a couple of units higher, depending on the ambient salinity conditions (Quast and Howe, 1980; Partridge et al., 2007). This suggests that the here applied high salinity treatment is rather hyper- to isosmotic, whereas the low salinity treatment is hypoosmotic. The hormone prolactin is involved in many metabolic pathways in vertebrates and highly relevant for fish in hypoosmotic conditions as it prevents the loss of ions and the uptake of water. Both mechanisms are crucial in hypoosmotic conditions to maintain homeostasis (McCormick, 2001; Manzon, 2002; Breves et al., 2014). In our study, prolactin (*prl*) was the gene with the strongest upregulation in juveniles at low developmental salinity conditions underlining the ability of pipefish to quickly respond to prevailing salinity conditions. Similar patterns in the upregulation of prolactin in marine fish have been identified in black porgy *Acanthopagrus schlegelii* (Tomy et al., 2009) and rainbow trout *Oncorhynchus mykiss* (Prunet et al., 1990). This implies that higher *prl* expression under low salinity conditions could be indicative for adaptive developmental plasticity and suggest that juvenile fish are able to cope with short term salinity changes.

Some strains of the species *Vibrio alginolyticus* have been shown to become more virulent under low saline conditions (Dayma et al., 2015; Poirier et al., 2017). Drivers for this increased virulence can be trade-offs in the host (Birrer et al., 2012; Poirier et al., 2017), a phenotypic response of the bacteria (Hase and Barquera, 2001; Dayma et al., 2015) or a genetic adaptation of the bacteria to low salinity (Brown et al., 2012). Under low saline condition, we thus expected strong selection for immunological adaptation toward the prevailing pathogens that potentially resulted in a higher tolerance or a more effective immune defense against *Vibrio* bacteria. In line with this expectation, we found that pipefish offspring from parents caught at low origin salinity survived better when exposed to *Vibrio* bacteria than offspring from parents caught at high saline origins. This suggests that local adaptation to low saline conditions allows pipefish to allocate sufficient resources toward their immune system for fighting *Vibrio* infections. To this end, we found support for our hypothesis that increased *Vibrio* virulence in marine host organism can result from resource allocation tradeoffs toward osmoregulation, impairing the host's immune system (Birrer et al., 2012).

The bacteria used in this experiment were previously evolved at the respective high (v15: 15 PSU) or low (v7: 7 PSU) Baltic

Sea salinity condition. If genetic adaptation of bacteria to low salinity induces their virulence, we would have expected that the bacteria evolved at 7 PSU (v7) are more virulent, in particular for the pipefish offspring from parents originating from high saline locations. In contrast to our expectation, we have identified that v15 caused a higher mortality in juveniles originating from a high saline environment than in juveniles coming from low origin salinity and low parental salinity acclimation, while the impact of v7 was not differentiable across all groups. Gene expression measurements were not appropriate to answer the question of induced virulence and a corresponding stronger host immune response against bacteria evolved at low or high salinity depending on pipefish local salinity adaptation. Instead, the injury imposed by the injection had the strongest impact on the gene expression pattern. This implies that we did not identify a difference in gene expression of sham-injected animals and those animals injected either with v7 or v15. This is an unexpected limitation of our study. However, given that *Vibrio* bacteria had an impact on pipefish mortality, we assume that we have simply not chosen the time point when immune reactions against the *Vibrio* infection would have been best mirrored in the gene expression patterns but rather the timepoint when inflammation or stress responses induced by the injection were strongest. Bearing these limitations in mind, in combination with the higher survival of juveniles originating from low salinity parents, we suggest that the increased virulence of the *V. alginolyticus* strain is mainly driven by trade-offs impairing the pipefish's immune system. A deficiency that can potentially be overcome by local adaptation.

The patterns identified here have to be interpreted with care. Due to the unintended brood pouch infection that negatively affected 47% of the pregnant males originating from high saline conditions and parentally acclimated at low saline conditions, we are dealing with distinct selection intensities on the different treatment groups (Roth et al., 2018). In the treatment affected by the brood pouch fungus (Origin Salinity: *H*, Acclimation salinity: L) multiple clutches have at least been partially lost and potentially all infected fathers were suffering stress levels that can seriously confound the results from this study. The brood pouch fungus has severely impacted offspring development such that only the strongest will have survived. Addressing life history traits in the offspring and their gene expression will thus only be done in the strongest animals within the *HL* group, which does not resemble the original cohort, and makes interpretation of the data in the offspring generation difficult. We are aware of this limitation and have been taking this into account when interpreting our data.

CONCLUSION

After the last glacial maximum, broad-nosed pipefish have successfully populated the low salinity areas of the Baltic Sea. The results of our study suggest that the components of this success story are a mixture of genetic adaptation and the maintenance of a high degree of phenotypic plasticity of locally adapted pipefish enabling them to deal with present and ancestral salinity levels and re-occurring salinity fluctuations. Future genome scans will give closer insights into local adaptation of the pipefish to the salinity gradient of the Baltic Sea. Evidence for adaptive *trans*-generational effects were limited to expression changes in adaptive immune genes. Pipefish individuals with suitable alleles for low salinity conditions can inhabit low saline environments. The adaptation and adjustment of life-history strategies to lower salinity also enable pipefish to cope with prevailing pathogens such as *Vibrio* bacteria or aquatic fungi. Pipefish of the species *S. typhle* inhabiting the Baltic Sea are thus expected to genetically adapt to predicted further drops in salinity and retain phenotypic plasticity to cope with temporarily varying salinity levels. The findings are underlined by the fact that *S. typhle* also inhabit areas in the northern part of the Baltic Sea with salinity levels below 5 PSU.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the online repository PANGAEA: https://doi.pangaea.de/10.1594/PANGAEA.926923.

ETHICS STATEMENT

The animal study was reviewed and approved by the Experimental work was conducted in agreement with the German animal welfare law and approved by the Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Digitalisierung under permission MELUR V 312–7224.121-19 (67–5/13), "komparative Vergleichsstudie von Immunantwort-Transfer von Eltern zu Nachkommen in Fischarten mit extremer Brutpflege"). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

OR and HG designed the study with input from CW. HG, K-SW, and OR collected fish in the field, conducted the experiment, and sampled the fish. LS designed the primers. LS, HG, and K-SW did the molecular lab work. HG, LS, OR, and K-SW analyzed the data. HG, LS, and OR wrote the manuscript with input from all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.626442/full#supplementary-material

REFERENCES

Akaike, H. (1976). An information criteron (AIC). *Math. Sci.* 14, 5–7.

Andersson, A., Meier, H. E. M., Ripszam, M., Rowe, O., Wikner, J., Haglund, P., et al. (2015). Projected future climate change and Baltic Sea ecosystem management. *Ambio* 44, 345–356. doi: 10.1007/s13280-015-0654-8

Angers, B., Castonguay, E., and Massicotte, R. (2010). Environmentally induced phenotypes and DNA methylation: how to deal with unpredictable conditions until the next generation and after. *Mol. Ecol.* 19, 1283–1295. doi: 10.1111/j.1365-294x.2010.04580.x

Baker-Austin, C., Trinanes, J., Gonzalez-Escalona, N., and Martinez-Urtaza, J. (2017). Non-cholera vibrios: the microbial barometer of climate change. *Trends Microbiol.* 25, 76–84. doi: 10.1016/j.tim.2016.09.008

Barrett, R. D. H., and Schluter, D. (2008). Adaptation from standing genetic variation. *Trends Ecol. Evol.* 23, 38–44. doi: 10.1016/j.tree.2007.09.008

Beemelmanns, A., and Roth, O. (2016a). Bacteria-type-specific biparental immune priming in the pipefish *Syngnathus typhle*. *Ecol. Evol.* 6, 6735–6757. doi: 10.1002/ece3.2391

Beemelmanns, A., and Roth, O. (2016b). Biparental immune priming in the pipefish *Syngnathus typhle*. *Zoology* 119, 262–272. doi: 10.1016/j.zool.2016.06.002

Beemelmanns, A., and Roth, O. (2017). Grandparental immune priming in the pipefish *Syngnathus typhle*. *BMC Evol. Biol.* 17:44. doi: 10.1186/s12862-017-0885-3

Belkhir, K. (2004). *Genetix 4.05, logiciel sous Windows TM pour la Genetique des Populations*. Available online at: http://www.genetix.univ-montp2.fr/genetix/genetix.htm (accessed June 22, 2018).

Berg, P. R., Jentoft, S., Star, B., Ring, K. H., Knutsen, H., Lien, S., et al. (2015). Adaptation to low salinity promotes genomic divergence in atlantic cod (*Gadus morhua* L.). *Genome Biol. Evol.* 7, 1644–1663. doi: 10.1093/gbe/evv093

Berglund, A., Sundin, J., and Rosenqvist, G. (2017). Baltic pipefish females need twice as many males as they get. *Behav. Ecol.* 28, 827–832. doi: 10.1093/beheco/arx046

Birrer, S. C., Reusch, T. B. H., and Roth, O. (2012). Salinity change impairs pipefish immune defence. *Fish Shellfish Immunol.* 33, 1238–1248. doi: 10.1016/j.fsi.2012.08.028

Björck, S. (1995). A review of the history of the Baltic Sea, 13.0-8.0 ka BP. *Quat. Int.* 27, 19–40. doi: 10.1016/1040-6182(94)00057-c

Blanquart, F., and Gandon, S. (2013). Time-shift experiments and patterns of adaptation across time and space. *Ecol. Lett.* 16, 31–38. doi: 10.1111/ele.12007

Blois, J. L., Williams, J. W., Fitzpatrick, M. C., Jackson, S. T., and Ferrier, S. (2013). Space can substitute for time in predicting climate-change effects on biodiversity. *Proc. Natl. Acad. Sci. U.S.A.* 110, 9374–9379. doi: 10.1073/pnas.1220228110

Bock, G., and Lieberum, C. (2017). Neobiota in Ausgewählten Häfen der Schleswig-Holsteinischen Ostsee, Kiel: Landesamtes für Landwirtschaft, Umwelt und ländliche Räume des Landes Schleswig-Holstein. doi: 10.1073/pnas.1220228110

Boeuf, G., and Payan, P. (2001). How should salinity influence fish growth? *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 130, 411–423. doi: 10.1016/s1532-0456(01)00268-x

Bookout, A. L., and Mangelsdorf, D. J. (2003). Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. *Nuclear Recept. Signal.* 1:e012.

Boyett, H. V., Bourne, D. G., and Willis, B. L. (2007). Elevated temperature and light enhance progression and spread of black band disease on staghorn corals of the Great Barrier Reef. *Mar. Biol.* 151, 1711–1720. doi: 10.1007/s00227-006-0603-y

Breves, J. P., McCormick, S. D., and Karlstrom, R. O. (2014). Prolactin and teleost ionocytes: new insights into cellular and molecular targets of prolactin in vertebrate epithelia. *Gen. Comp. Endocrinol.* 203, 21–28. doi: 10.1016/j.ygcen.2013.12.014

Brierley, A. S., and Kingsford, M. J. (2009). Impacts of climate change on marine organisms and ecosystems. *Curr. Biol.* 19, 602–614.

Brown, S. P., Cornforth, D. M., and Mideo, N. (2012). Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. *Trends Microbiol.* 20, 336–342. doi: 10.1016/j.tim.2012.04.005

Brunner, F. S., and Eizaguirre, C. (2016). Can environmental change affect host/parasite-mediated speciation? *Zoology* 119, 384–394. doi: 10.1016/j.zool.2016.04.001

Chatterjee, K., Pavlogiannis, A., Adlam, B., and Nowak, M. A. (2014). The time scale of evolutionary innovation. *PLoS Comput. Biol.* 10:e1003818. doi: 10.1371/journal.pcbi.1003818

Chen, M. X., Li, H. Y., Li, G., and Zheng, T. L. (2011). Distribution of Vibrio alginolyticus-like species in shenzhen coastal waters, China. *Braz. J. Microbiol.* 42, 884–896. doi: 10.1590/s1517-83822011000300007

Chevin, L. M., Lande, R., and Mace, G. M. (2010). Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. *PLoS Biology* 8: e1000357. doi: 10.1371/journal.pbio.1000357

Chibani, C. M., Hertel, R., Hoppert, M., Liesegang, H., and Wendling, C. C. (2020a). Closely related vibrio alginolyticus strains encode an identical repertoire of caudovirales-like regions and filamentous phages. *Viruses* 12:1359. doi: 10.3390/v12121359

Chibani, C. M., Roth, O., Liesegang, H., and Wendling, C. C. (2020b). Genomic variation among closely related *Vibrio alginolyticus* strains is located on mobile genetic elements. *BMC Genomics* 21:354. doi: 10.1186/s12864-020-6735-5

Dayma, P. I, Raval, H., Joshi, N., Patel, N. P., Haldar, S., and Mody, K. H. (2015). Influence of low salinity stress on virulence and biofilm formation potential in Vibrio alginolyticus, isolated from the Gulf of Khambhat, Gujarat India. *Aquat. Living Resour.* 28, 99–109. doi: 10.1051/alr/2016004

DeFaveri, J., and Merila, J. (2014). Local adaptation to salinity in the three-spined stickleback? *J. Evol. Biol.* 27, 290–302. doi: 10.1111/jeb.12289

DeWitt, T. J., Sih, A., and Wilson, D. S. (1998). Costs and limits of phenotypic plasticity. *Trends Ecol. Evol.* 13, 77–81. doi: 10.1016/s0169-5347(97)01274-3

Donelson, J. M., Sunday, J. M., Figueira, W. F., Gaitan-Espitia, J. D., Hobday, A. J., Johnson, C. R., et al. (2019). Understanding interactions between plasticity,

adaptation and range shifts in response to marine environmental change. *Philos. Trans. R. Soc. B Biol. Sci.* 374, 14.

Dray, S., and Dufour, A.-B. (2007). The ade4 package: implementing the duality diagram for ecologists. *J. Stat. Softw.* 22:15378.

Dutil, J. D., Lambert, Y., and Boucher, E. (1997). Does higher growth rate in Atlantic cod (*Gadus morhua*) at low salinity result from lower standard metabolic rate or increased protein digestibility? *Can. J. Fish. Aquat. Sci.* 54, 99–103. doi: 10.1139/f96-148

Eizaguirre, C., Lenz, T. L., Kalbe, M., and Milinski, M. (2012). Rapid and adaptive evolution of MHC genes under parasite selection in experimental vertebrate populations. *Nat. Commun.* 3:621.

Eriksen, M. S., Bakken, M., Espmark, A., Braastad, B. O., and Salte, R. (2006). Prespawning stress in farmed Atlantic salmon Salmo salar: maternal cortisol exposure and hyperthermia during embryonic development affect offspring survival, growth and incidence of malformations. *J. Fish Biol.* 69, 114–129. doi: 10.1111/j.1095-8649.2006.01071.x

Felsenstein (1989). *PHYLIP-Phylogeny Inference Package (Ver. 3.2), Cladistics*.

Fraser, H. B. (2013). Gene expression drives local adaptation in humans. *Genome Res.* 23, 1089–1096. doi: 10.1101/gr.152710.112

Gibson, J. R., and Najjar, R. G. (2000). The response of Chesapeake Bay salinity to climate-induced changes in streamflow. *Limnol. Oceanogr.* 45, 1764–1772. doi: 10.4319/lo.2000.45.8.1764

Gienapp, P., Teplitsky, C., Alho, J. S., Mills, J. A., and Merila, J. (2008). Climate change and evolution: disentangling environmental and genetic responses. *Mol. Ecol.* 17, 167–178. doi: 10.1111/j.1365-294x.2007.03413.x

Guo, B. C., DeFaveri, J., Sotelo, G., Nair, A., and Merila, J. (2015). Population genomic evidence for adaptive differentiation in Baltic Sea three-spined sticklebacks. *BMC Biol.* 13:19. doi: 10.1186/s12915-015-0130-8

Guo, B. C., Li, Z. T., and Merila, J. (2016). Population genomic evidence for adaptive differentiation in the Baltic Sea herring. *Mol. Ecol.* 25, 2833–2852. doi: 10.1111/mec.13657

Gurkan, S., and Taskavak, E. (2007). Length-weigth relationships for syngnathid fishes of the Aegean Sea, Turkey. *Belgian J. Zool.* 137, 219–222.

Haddy, J. A., and Pankhurst, N. W. (2000). The effects of salinity on reproductive development, plasma steroid levels, fertilisation and egg survival in black bream *Acanthopagrus butcheri*. *Aquaculture* 188, 115–131. doi: 10.1016/s0044-8486(00)00326-4

Hall, M. D., Vettiger, A., and Ebert, D. (2013). Interactions between environmental stressors: the influence of salinity on host-parasite interactions between *Daphnia magna* and *Pasteuria ramosa*. *Oecologia* 171, 789–796. doi: 10.1007/s00442-012-2452-3

Hasan, M. M., DeFaveri, J., Kuure, S., Dash, S. N., Lehtonen, S., Merila, J., et al. (2017). Sticklebacks adapted to divergent osmotic environments show differences in plasticity for kidney morphology and candidate gene expression. *J. Exp. Biol.* 220, 2175–2186. doi: 10.1242/jeb.146027

Hase, C. C., and Barquera, B. (2001). Role of sodium bioenergetics in *Vibrio cholerae*. *Biochim. Biophys. Acta Bioenerg.* 1505, 169–178. doi: 10.1016/s0005-2728(00)00286-3

Heckwolf, M. J., Meyer, B. S., Doring, T., Eizaguirre, C., and Reusch, T. B. H. (2018). Transgenerational plasticity and selection shape the adaptive potential of sticklebacks to salinity change. *Evol. Appl.* 11, 1873–1885. doi: 10.1111/eva.12688

Heckwolf, M. J., Meyer, B. S., Hasler, R., Hoppner, M. P., Eizaguirre, C., and Reusch, T. B. H. (2020). Two different epigenetic information channels in wild three-spined sticklebacks are involved in salinity adaptation. *Sci. Adv.* 6:13.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8:19.

Hinrichs, I., Jahnke-Bornemann, A., Andersson, A., Ganske, A., Gouretski, V., Jensen, C., et al. (2019). The Baltic and North Seas Climatology (BNSC) – a comprehensive, observation-based data product of atmospheric and hydrographic parameters. *Front. Earth Sci.* 7:158. doi: 10.3389/feart.2019.00158

Holmborn, T., Goetze, E., Pollupuu, M., and Pollumae, A. (2011). Genetic species identification and low genetic diversity in *Pseudocalanus acuspes* of the Baltic Sea. *J. Plankton Res.* 33, 507–515. doi: 10.1093/plankt/fbq113

Hothorn, T., Bretz, F., Westfall, P., Heiberger, R. M., Schetzenmeister, A., and Scheibe, S. (2020). *multcomp: Simultaneous Inference in General Parametric Models*. Available online at: https://cran.r-project.org/web/packages/multcomp/index.html (accessed June 29, 2020).

Hulce, D., Li, X., Snyder-Leiby, T., and Johathan Liu, C. S. (2011). GeneMarker® genotyping software: tools to increase the statistical power of DNA fragment analysis. *J. Biomol. Tech.* 22, S35–S36.

Ilyina, T. S. (2015). Filamentous bacteriophages and their role in the virulence and evolution of pathogenic bacteria. *Mol. Genet. Microbiol. Virol.* 30, 1–9. doi: 10.3103/s0891416815010036

Imsland, A. K., Foss, A., Gunnarsson, S., Berntssen, M. H. G., FitzGerald, R., Bonga, S. W., et al. (2001). The interaction of temperature and salinity on growth and food conversion in juvenile turbot (*Scophthalmus maximus*). *Aquaculture* 198, 353–367. doi: 10.1016/s0044-8486(01)00507-5

Janeway, C. (2005). *Immunobiology: The Immune System in Health And Disease*. New York, NY: Garland Science.

Janssen, F., Schrum, C., and Backhaus, J. O. (1999). A climatological data set of temperature and salinity for the Baltic Sea and the North Sea. *Deutsche Jydrpgrafische Zeitschrift* 51:5. doi: 10.1007/bf02933676

Johannesson, K., and Andre, C. (2006). Life on the margin: genetic isolation and diversity loss in a peripheral marine ecosystem, the Baltic Sea. *Mol. Ecol.* 15, 2013–2029. doi: 10.1111/j.1365-294x.2006.02919.x

Johannesson, K., Le Moan, A., Perini, S., and André, C. (2020). A Darwinian laboratory of multiple contact zones. *Trends Ecol. Evol.* 35, 1021–1036. doi: 10.1016/j.tree.2020.07.015

Jones, A. G., Rosenqvist, G., Berglund, A., and Avise, J. C. (1999). The genetic mating system of a sex-role-reversed pipefish (*Syngnathus typhle*): a molecular inquiry. *Behav. Ecol. Sociobiol.* 46, 357–365. doi: 10.1007/s002650050630

Kelly, M. (2019). Adaptation to climate change through genetic accommodation and assimilation of plastic phenotypes. *Philos. Trans. R. Soc. B Biol. Sci.* 374:10.

Kim, L. H., and Chong, T. H. (2017). Physiological responses of salinity-stressed *Vibrio* sp and the effect on the biofilm formation on a nanofiltration membrane. *Environ. Sci. Technol.* 51, 1249–1258. doi: 10.1021/acs.est.6b02904

Kniebusch, M., Meier, H. E. M., and Radtke, H. (2019). Changing salinity gradients in the Baltic Sea as a consequence of altered freshwater budgets. *Geophys. Res. Lett.* 46, 9739–9747. doi: 10.1029/2019gl083902

Koressaar, T., and Remm, M. (2007). Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23, 1289–1291. doi: 10.1093/bioinformatics/btm091

Kutzer, M. A. M., and Armitage, S. A. O. (2016). Maximising fitness in the face of parasites: a review of host tolerance. *Zoology* 119, 281–289. doi: 10.1016/j.zool.2016.05.011

Laland, K., Uller, T., Feldman, M., Sterelny, K., Muller, G. B., Moczek, A., et al. (2014). Does evolutionary theory need a rethink? - POINT Yes, urgently. *Nature* 514, 161–164. doi: 10.1038/514161a

Larsen, P. F., Schulte, P. M., and Nielsen, E. E. (2011). Gene expression analysis for the identification of selection and local adaptation in fishes. *J. Fish Biol.* 78, 1–22. doi: 10.1111/j.1095-8649.2010.02834.x

Leder, E. H., Andr, C., Le Moan, A., Topel, M., Blomberg, A., Havenhand, J. N., et al. (2021). Post-glacial establishment of locally adapted fish populations over a steep salinity gradient. *J. Evol. Biol.* 34, 138–156.

Lehtonen, T. K., and Kvarnemo, C. (2015). Infections may select for filial cannibalism by impacting egg survival in interactions with water salinity and egg density. *Oecologia* 178, 673–683. doi: 10.1007/s00442-015-3246-1

Lind, M. I., Zwoinska, M. K., Andersson, J., Carlsson, H., Krieg, T., Larva, T., et al. (2020). Environmental variation mediates the evolution of anticipatory parental effects. *Evol. Lett.* 4, 371–381. doi: 10.1002/evl3.177

Loder, J. W., van der Baaren, A., and Yashayaev, I. (2015). Climate comparisons and change projections for the Northwest Atlantic from six CMIP5 models. *Atmosphere Ocean* 53, 529–555. doi: 10.1080/07055900.2015.1087836

Manzon, L. A. (2002). The role of prolactin in fish osmoregulation: a review. *Gen. Comp. Endocrinol.* 125, 291–310. doi: 10.1006/gcen.2001.7746

Marshall, D. J. (2008). Transgenerational plasticity in the sea: context-dependent maternal effects across the life history. *Ecology* 89, 418–427. doi: 10.1890/07-0449.1

McCormick, S. D. (2001). Endocrine control of osmoregulation in teleost fish. *Am. Zool.* 41, 781–794. doi: 10.1093/icb/41.4.781

McGuigan, K., Nishimura, N., Currey, M., Hurwit, D., and Cresko, W. A. (2011). Cryptic genetic variation and body size evolution in threespine stickleback. *Evolution* 65, 1203–1211. doi: 10.1111/j.1558-5646.2010.01195.x

Meier, H. E. M., Kjellstrom, E., and Graham, L. P. (2006). Estimating uncertainties of projected Baltic Sea salinity in the late 21st century. *Geophys. Res. Lett.* 33:4.

Molnar, P. K., Kutz, S. J., Hoar, B. M., and Dobson, A. P. (2013). Metabolic approaches to understanding climate change impacts on seasonal host-macroparasite dynamics. *Ecol. Lett.* 16, 9–21. doi: 10.1111/ele.12022

Morgan, J. D., and Iwama, G. K. (1991). Effects of salinity on growth, metabolism, and ion regulation in juvenile rainbow and steelhead trout (*Oncorhynchus mykiss*) and fall chinook salmon (*Oncorhynchus tshawytscha*). *Can. J. Fish. Aquat. Sci.* 48, 2083–2094. doi: 10.1139/f91-247

Mostowy, R., and Engelstadter, J. (2011). The impact of environmental change on host-parasite coevolutionary dynamics. *Proc. R. Soc. B Biol. Sci.* 278, 2283–2292. doi: 10.1098/rspb.2010.2359

Mück, I., and Heubel, K. U. (2018). Ecological variation along the salinity gradient in the Baltic Sea Area and its consequences for reproduction in the common goby. *Curr. Zool.* 64, 259–270. doi: 10.1093/cz/zoy006

Murray, C. S., Malvezzi, A., Gobler, C. J., and Baumann, H. (2014). Offspring sensitivity to ocean acidification changes seasonally in a coastal marine fish. *Mar. Ecol. Prog. Series* 504, 1–11. doi: 10.3354/meps10791

Nissling, A., Johansson, U., and Jacobsson, M. (2006). Effects of salinity and temperature conditions on the reproductive success of turbot (*Scophthalmus maximus*) in the Baltic Sea. *Fish. Res.* 80, 230–238. doi: 10.1016/j.fishres.2006.04.005

Nissling, A., Nyberg, S., and Petereit, C. (2017). Egg buoyancy of flounder, *Platichthys flesus*, in the Baltic Sea–adaptation to salinity and implications for egg survival. *Fish. Res.* 191, 179–189. doi: 10.1016/j.fishres.2017.02.020

Nissling, A., and Westin, L. (1997). Salinity requirements for successful spawning of Baltic and Belt Sea cod and the potential for cod stock interactions in the Baltic Sea. *Mar. Ecol. Prog. Ser.* 152, 261–271. doi: 10.3354/meps152261

Nygård, M., Kvarnemo, C., Ahnesjö, I., and Braga Goncalves, I. (2019). Pipefish embryo oxygenation, survival, and development: egg size, male size, and temperature effects. *Behav. Ecol.* 30, 1451–1460. doi: 10.1093/beheco/arz101

Oberbeckmann, S., Wichels, A., Wiltshire, K. H., and Gerdts, G. (2011). Occurrence of Vibrio parahaemolyticus and Vibrio alginolyticus in the German Bight over a seasonal cycle. *Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* 100, 291–307. doi: 10.1007/s10482-011-9586-x

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2019). *vegan: Community Ecology Package. R packae version 2.5-4*.

Oosterhout, C. V., Hutchinson, W. F., Wills, D. P. M., and Shipley, P. (2004). micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* 4, 535–538. doi: 10.1111/j.1471-8286.2004.00684.x

Partridge, C., Shardo, J., and Boettcher, A. (2007). Osmoregulatory role of the brood pouch in the euryhaline *Gulf pipefish*, *Syngnathus scovelli*. *Comp. Biochem. Physiol.* 147, 556–561. doi: 10.1016/j.cbpa.2007.02.007

Poirier, M., Listmann, L., and Roth, O. (2017). Selection by higher-order effects of salinity and bacteria on early life-stages of Western Baltic spring-spawning herring. *Evol. Appl.* 10, 603–615. doi: 10.1111/eva.12477

Poloczanska, E. S., Brown, C. J., Sydeman, W. J., Kiessling, W., Schoeman, D. S., Moore, P. J., et al. (2013). Global imprint of climate change on marine life. *Nat. Clim. Change* 3, 919–925.

Pritchard, J. K., Stephens, M., and Donnelly, P. (2000). Inference of population structure using Multilocus genotype data. *Genetics* 155, 945.

Prunet, P., Avelia, M., Föstier, A., Björnsson, B. T., Boeuf, G., and Haux, C. (1990). Roles of prolactin in salmonids. *Prog. Comp. Endocrinol.* 342, 547–552.

Quast, W. D., and Howe, N. R. (1980). The osmotic role of the brood pouch in the pipefish *Syngnathus scovelli*. *Comp. Biochem. Physiol. Physiol.* 67, 675–678. doi: 10.1016/0300-9629(80)90259-5

RCoreTeam (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Reed, T. E., Waples, R. S., Schindler, D. E., Hard, J. J., and Kinnison, M. T. (2010). Phenotypic plasticity and population viability: the importance of environmental predictability. *Proc. R. Soc. B Biol. Sci.* 277, 3391–3400. doi: 10.1098/rspb.2010.0771

Reusch, T. B. H., Dierking, J., Andersson, H. C., Bonsdorff, E., Carstensen, J., Casini, M., et al. (2018). The Baltic Sea as a time machine for the future coastal ocean. *Sci. Adv.* 4:eaar8195.

Rispoli, V. F., and Wilson, A. B. (2008). Sexual size dimorphism predicts the frequency of multiple mating in the sex-role reversed pipefish *Syngnathus typhle*. *J. Evol. Biol.* 21, 30–38. doi: 10.1111/j.1420-9101.2007.01470.x

Rolfe, D. F. S., and Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol. Rev.* 77, 731–758. doi: 10.1152/physrev.1997.77.3.731

Roth, O., Beemelmanns, A., Barribeau, S. M., and Sadd, B. M. (2018). Recent advances in vertebrate and invertebrate transgenerational immunity in the light of ecology and evolution. *Heredity* 121, 225–238. doi: 10.1038/s41437-018-0101-2

Roth, O., Keller, I., Landis, S. H., Salzburger, W., and Reusch, T. B. H. (2012a). Host are ahead in a marine host-parasite coevolutionary arms race: innate ummune system adapatation in pipefish *Syngnathus typhle* agains Vibrio phylotypes. *Evolution* 66, 2528–2539. doi: 10.1111/j.1558-5646.2012.01614.x

Roth, O., Klein, V., Beemelmanns, A., Scharsack, J. P., and Reusch, T. B. H. (2012b). Male pregnancy and biparental immune priming. *Am. Nat.* 180, 802–814. doi: 10.1086/668081

Roth, O., and Landis, S. H. (2017). Trans-generational plasticity in response to immune challenge is constrained by heat stress. *Evol. Appl.* 10, 514–528. doi: 10.1111/eva.12473

Schaarschmidt, T., Meyer, E., and Jurss, K. (1999). A comparison of transport-related gill enzyme activities and tissue-specific free amino acid concentrates of Baltic Sea (brackish water) and freshwater threespine sticklebacks, *Gasterosteus aculeatus*, after salinity and temperature acclimation. *Mar. Biol.* 135, 689–697. doi: 10.1007/s002270050670

Schneider, R. F., and Meyer, A. (2017). How plasticity, genetic assimilation and cryptic genetic variation may contribute to adaptive radiations. *Mol. Ecol.* 26, 330–350. doi: 10.1111/mec.13880

Shama, L. N. S., and Wegner, K. M. (2014). Grandparental effects in marine sticklebacks: transgenerational plasticity across multiple generations. *J. Evol. Biol.* 27, 2297–2307. doi: 10.1111/jeb.12490

Stockwell, C. A., Purcell, K. M., Collyer, M. L., and Janovy, J. (2011). Effects of salinity on *Physa acuta*, the intermediate host for the parasite *Posthodiplostomum minimum*: implications for the translocation of the protected white sands pupfish. *Trans. Am. Fish. Soc.* 140, 1370–1374. doi: 10.1080/00028487.2011.620499

Sullivan, T. J., and Neigel, J. E. (2018). Effects of temperature and salinity on prevalence and intensity of infection of blue crabs, *Callinectes sapidus*, by *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus* in Louisiana. *J. Invertebr. Pathol.* 151, 82–90. doi: 10.1016/j.jip.2017.11.004

Sunde, J., Yildirim, Y., Tibblin, P., and Forsman, A. (2020). Comparing the performance of microsatellites and RADseq in population genetic studies: analysis of data for pike (*Esox lucius*) and a synthesis of previous studies. *Front. Genet.* 11:218. doi: 10.3389/fgene.2020.00218

Sunday, J. M., Calosi, P., Dupont, S., Munday, P. L., Stillman, J. H., and Reusch, T. B. H. (2014). Evolution in an acidifying ocean. *Trends Ecol. Evol.* 29, 117–125. doi: 10.1016/j.tree.2013.11.001

Swaggerty, C. L. I, Pevzner, Y., Kaiser, P., and Kogut, M. H. (2008). Profiling pro-inflammatory cytokine and chemokine mRNA expression levels as a novel method for selection of increased innate immune responsiveness. *Vet. Immunol. Immunopathol.* 126, 35–42. doi: 10.1016/j.vetimm.2008.06.005

Tomy, S., Chang, Y. M., Chen, Y. H., Cao, J. C., Wang, T. P., and Chang, C. F. (2009). Salinity effects on the expression of osmoregulatory genes in the euryhaline black porgy *Acanthopagrus schlegeli*. *Gen. Comp. Endocrinol.* 161, 123–132. doi: 10.1016/j.ygcen.2008.12.003

Torda, G., Donelson, J. M., Aranda, M., Barshis, D. J., Bay, L., Berumen, M. L., et al. (2017). Rapid adaptive responses to climate change in corals. *Nat. Clim. Change* 7, 627–636. doi: 10.1038/nclimate3374

Uller, T., Nakagawa, S., and English, S. (2013). Weak evidence for anticipatory parental effects in plants and animals. *J. Evol. Biol.* 26, 2161–2170. doi: 10.1111/jeb.12212

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., et al. (2012). Primer3–new capabilities and interfaces. *Nucleic Acids Res.* 40:e115. doi: 10.1093/nar/gks596

Urban, M. C. (2015). Accelerating extinction risk from climate change. *Science* 348, 571–573. doi: 10.1126/science.aaa4984

Ustups, D., Müller-Karulis, B., Bergstrom, U., Makarchouk, A., and Sics, I. (2013). The influence of environmental conditions on early life stages of flounder (*Platichthys flesus*) in the central Baltic Sea. *J. Sea Res.* 75, 77–84. doi: 10.1016/j.seares.2012.05.001

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:12.

Velasco, J., Gutierrez-Canovas, C., Botella-Cruz, M., Sanchez-Fernandez, D., Arribas, P., Carbonell, J. A., et al. (2019). Effects of salinity changes on aquatic organisms in a multiple stressor context. *Philos. Trans. R. Soc. B Biol. Sci.* 374:9.

Waldor, M. K., and Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* 272, 1910–1914. doi: 10.1126/science.272.5270.1910

Wendling, C. C., Piecyk, A., Refardt, D., Chibani, C., Hertel, R., Liesegang, H., et al. (2017). Tripartite species interaction: eukaryotic hosts suffer more from phage susceptible than from phage resistant bacteria. *BMC Evolutionary Biology* 17:98. doi: 10.1186/s12862-017-0930-2

West-Eberhard, M. J. (1989). Phenotypic plasticity and the origins of diversity. *Annu. Rev. Ecol. Syst.* 20, 249–278.

Wilson, A. B., and Veraguth, I. E. (2010). The impact of Pleistocene glaciation across the range of a widespread European coastal species. *Mol. Ecol.* 19, 4535–4553. doi: 10.1111/j.1365-294x.2010.04811.x

Wilson, A. B., Wegmann, A., Ahnesjo, I., and Goncalves, J. M. S. (2020). The evolution of ecological specialization across the range of a broadly distributed marine species. *Evolution* 74, 629–643. doi: 10.1111/evo.13930

Yuan, J. S., Reed, A., Chen, F., and Stewart, C. N. Jr. (2006). Statistical analysis of real-time PCR data. *BMC Bioinform.* 7:85. doi: 10.1186/1471-2105-7-85

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Transgenerational Effects of Parental Diet on Offspring Development and Disease Resistance in Flies

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The environmental conditions experienced by parents influence next generations, with the parental nutritional status playing an important role in shaping offspring phenotypes. Our understanding of transgenerational effects of parental diet on offspring pathogen resistance is, however, poorly documented. We manipulated the quality of parental diet (i.e., mother, father, or both) and measured effects on offspring development and survival after an immune challenge by septic infection. We used *Bactrocera tryoni* as host model infected with the pathogenic bacterium, *Serratia marcescens*. Our results showed no significant effect of maternal, or paternal, diet on offspring resistance. Interestingly, when the diet of both parents was manipulated, sons from parents fed either carbohydrate- or protein-biased diets had higher survival upon pathogen infection than sons from parents fed balanced diets. The quality of the parental diet had no effect on offspring developmental traits with the exception of egg hatching percentage which decreased when mothers were fed a protein-biased diet. Our results emphasised the complexity of nutritional transgenerational effects on offspring pathogen resistance and development.

Keywords: transgenerational effects, parental diet, *Serratia marcescens*, offspring, development, disease resistance

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INTRODUCTION

Environmental conditions experienced by parents can have long-term effects (Burton and Metcalfe, 2014; Woestmann and Saastamoinen, 2016). In both vertebrates and invertebrates, parents in good physiological conditions (e.g., good nutritional condition or favourable temperature) tend to produce offspring with higher fitness relative to the offspring of parents with low physiological conditions (Mousseau and Fox, 1998; Qvarnström and Price, 2001; Bonduriansky and Head, 2007). The environmental conditions experienced by parents can also serve as a cue to predict conditions that offspring are likely to experience and offspring can gain benefits from acquiring information of their future environment (i.e., "transgenerational phenotypic plasticity") (West-Eberhard, 2003; Pigliucci, 2001; Whitman and Agrawal, 2009). The outcome of transgenerational phenotypic plasticity depends, however, on whether environmental conditions experienced across generations are predictable or not (Burgess and Marshall, 2014) and, therefore, if the parental environment is a reliable predictor of the offspring environment (e.g., Gluckman and Hanson, 2004; Galloway and Etterson, 2007; Marshall and Uller, 2007; Bateson et al., 2014; Burgess and Marshall, 2014; Murren et al., 2015). A mismatch between the offspring' and parents' environments

can potentially reduce offspring fitness (see e.g., Gluckman et al., 2007; Raubenheimer et al., 2012), although this is not true for all taxa (Uller et al., 2013).

Amongst the key environmental factors experienced by parents, nutrition is an essential factor that shapes offspring's phenotype (Bonduriansky and Day, 2009). In insects, parental diet influences many offspring traits including body size and body weight (Vijendravarma et al., 2010; Triggs and Knell, 2012; Valtonen et al., 2012; Dew-Budd et al., 2016), developmental time (Vijendravarma et al., 2010; Valtonen et al., 2012; Matzkin et al., 2013), egg-to-adult survivorship (Prasad et al., 2003), egg hatching success (Bonduriansky et al., 2016), reproduction (Matzkin et al., 2013) and body composition (Buescher et al., 2013; Matzkin et al., 2013; Brookheart and Duncan, 2016; Dew-Budd et al., 2016). Parental diet can also affect the transcriptome of offspring through a decrease in the quantity of ribosomal RNA and changes in expression levels of metabolic and chromatin-coding genes, which might result in cancers and diabetes in the offspring (Buescher et al., 2013; Öst et al., 2014; Aldrich and Maggert, 2015).

Immunity and resistance to infections are also known to be influenced by the quality of the parental diet. In *Drosophila melanogaster*, expression level of immune genes is lower in sons of fathers fed low-protein diets compared to sons of fathers fed high-protein diets (Zajitschek et al., 2017). When offspring are immune-challenged, parental diet also influence their immune response level and impact their resistance to infections. In the Indian meal moth *Plodia interpunctella*, for instance, larvae from mothers reared in poor diets (i.e., diets with high cellulose content) are less resistant to viral and fungal infections than larvae from parents reared in rich diets (i.e., diets with low cellulose content) (Boots and Roberts, 2012). This effect might be explained by a reduced phenoloxidase activity and lower number of haemocytes (Kangassalo et al., 2015), (Boots and Roberts, 2012), (Triggs and Knell, 2012). In the mosquito *Aedes aegypti,* daughters from parents kept on a poor diet have higher viral load compared to daughters from parents in a rich diet, an effect that persists even when the offspring are fed a rich diet (Zirbel et al., 2018). Nonetheless, no difference in adult survival after bacterial infection has been detected between offspring from parents fed standard (balanced) or protein-deprived diets (Valtonen et al., 2012). In the *Melitaea cinxia* butterfly, maternal diet (*ad libitum* vs. starvation) does not have a significant effect on the total number of haemocytes, granular cells and oenocytoids of offspring infected with the parasitoid *Cotesia melitaearum* (Saastamoinen et al., 2013). Together, these findings highlight the complexity of the effects of parental diet on offspring immunity and pathogen resistance.

To date, however, investigations in this field are still partial because manipulations of parental diet have mainly focussed on the total energy of the food, overlooking the effect of relative ratio between diet components on offspring traits. In addition, studies have mainly manipulated the diet of mothers or both parents simultaneously, overlooking the effects of fathers' diet. Yet, there is increasing evidence showing that the environment experienced by fathers can prime offspring basal immunity (Zanchi et al., 2011; Eggert et al., 2014) and increase offspring resistance to infection (Roth et al., 2010). As a result, we still lack a proper empirical investigation of the trans-generational effects of parental diet (both single-sex and both-sexes approaches) on immune state and resistance.

Here, we investigated transgenerational effects of nutrition by manipulating the protein-to-carbohydrate balance in the diet (hereafter referred to as "PC ratio") of mothers, fathers, and both parents simultaneously. The fruit fly *Bactrocera tryoni*, one of the most damaging pest insect in Australia (Hancock et al., 2000; Sutherst et al., 2000; Clarke et al., 2011), was used as model system. Transgenerational effects of diet manipulation were measured on (i) development traits including egg hatching percentage, pupation percentage, emergence percentage, larval weight, pupal weight and larval body lipid reserves and (ii) resistance to infection of the offspring once adult by recording the survival of male and female offspring after a septic infection with the pathogenic bacterium *Serratia marcescens*. Knowledge gained from this study contributes insights into ecological questions investigating the effects of parental environmental conditions on offspring life history traits. Results are discussed in the context of transgenerational phenotypic plasticity and what type of information parental diet can pass to the next generations.

MATERIALS AND METHODS

Fly Stock

Flies originated from a *B. tryoni* stock colony that was maintained on a gel-based larval diet (Moadeli et al., 2017) and raised as adult with *ad libitum* hydrolysed yeast (MP Biomedicals Cat. no 02103304) and sugar (CSR® White Sugar). The stock colony was maintained at 25°C and 65% humidity with a 12-h light/dark cycle for 25 generations.

Diet Preparation

We used four single liquid diets varying in the protein-to-carbohydrate ratio (PC 1:8, 1:5, 1:3, and 1:1) (Dinh et al., 2019). A choice diet was included as control where solutions of hydrolysed yeast and sugar were separately offered to the flies. Based on previous work, we considered PC 1:3 as a balanced diet for non-infected *B. tryoni* (see Fanson et al., 2009), PC 1:8 and 1:5 as carbohydrate-biased diets, and PC 1:1 as a protein-biased diet. We did not include any diets with a greater concentration of yeast because they would have had drastically affected fly mortality. Diets were prepared by mixing hydrolysed yeast (MP Biomedicals Cat. no 02103304) and/or sugar (CSR® White Sugar) in warm distilled water using a hot plate set at 80°C. All diets were made to a final concentration of 120 g/L. The hydrolysed yeast used in this study contains 62.1% protein and 1% carbohydrate.

Diet Manipulation in Parents

We performed three experiments where the diet of mothers, fathers, or both parents was manipulated, and the effects on offspring life-history traits were measured. Parental diet was manipulated only at adult stage. The experimental design is described in **Figure 1**. In the first experiment, the diet of mothers was manipulated by assigning groups of two-day old females to

one of five diet treatments (PC 1:8, 1:5, 1:3, 1:1, or choice diet), whereas, fathers were given a choice diet. In contrast, in the second experiment, the diet of fathers was manipulated (same treatments as above) while mothers were fed a choice diet. In the third experiment, we manipulated the diet of both parents (i.e., both parents were fed the same diet). Food was provided to the flies via a cotton ball soaked in the liquid diets. Flies from all experiments were fed the experimental diets for 12 days and allowed to mate in groups of 45 females and 45 males, selected randomly (noted that we set up the experiment with 60 males/females in order to obtain a final sample size of 45 males and females). Three mating cages were prepared for each diet treatment. Eggs were collected in each mating cage for 24 h using an ovipositional device that consisted in a plastic bottle with numerous puncture holes and filled with 5 mL of water to maintain humidity. All eggs were pooled and used to generate offspring for subsequent experiments.

Offspring Generation

Collected eggs were used in two experimental set ups.

(i) Measuring the percentage of egg hatching, percentage of pupation and percentage of emergence.

Groups of 100 eggs were randomly selected and transferred to 100 mm Petri-dishes containing 15 mL of standard larval diet (Moadeli et al., 2017). We recorded the number of eggs that did not hatch after 4 days. Petri-dishes with hatched eggs were then placed in 1.75 mL containers with 40 mL vermiculite where larvae, once they reached fourth instar, were allowed to jump out of their larval environment and pupate. We recorded the number of pupae and newly emerged flies. Two sets of eggs were collected from each mating cage, yielding six sets of eggs in total (100 eggs/set) collected from each diet treatment.

(ii) Measuring larval weight, pupal weight, larval body lipid, and pathogen resistance of adult offspring.

For this purpose, 90 μL of an egg solution (approximately 1,000 eggs mixed with water) was collected from each mating cage. Eggs were transferred to diet trays containing 150 mL of standard larval diet yielding in three replicates for each diet treatment (Moadeli et al., 2017). Five days after seeding the eggs, the trays were placed in 12.5 L containers with 500 mL vermiculite and lids removed to allow the larvae to jump and pupate. Twenty larvae per tray (60 in total per diet) were collected within 24 h since they started jumping out of the diet (7 days after seeding eggs) to measure larval body weight and body lipid. The rest of the larvae were left to develop. Seven days later, 20 pupae per tray (60 in total per diet) were collected for measurement of pupal body weight. The rest of pupae were allowed to develop into adults for the infection experiment. Upon emergence, adult flies were fed standard adult diet [(*ad libitum* hydrolysed yeast (MP Biomedicals Cat. no 02103304) and sugar (CSR® White Sugar)].

Larval Weight, Pupal Weight, and Larval Body Lipid

Larval and pupal weights were measured using a microbalance (Sartorius, accuracy ± 0.001mg).

Larval body lipid was measured in 21 individual larvae (3 replicates per diet treatment, 7 larvae/replicate) using the protocol described in Dinh et al. (2019). Briefly, larvae were snap frozen at −20°C, bodies transferred into individual 6 mL glass tubes (Sigma-Aldrich) and dried in a drying oven (Binder) at 50°C for 48 h. Dry body weight was measured using a microbalance (Sartorius, accuracy ± 0.001 mg). Total body lipid was then extracted in three 24 h washes of chloroform (Sigma-Aldrich Cat. No 650498). At the end of the third chloroform wash, lipid-free bodies were re-dried and re-weighed to calculate lipid content. The percentage of body lipid was calculated by subtracting the lipid-free dry body weight to the initial dry body weight and dividing the difference by the initial body weight multiplied by 100.

Survival of Adult Flies After Septic Infection With *Serratia marcescens*

Serratia marcescens (ATCC 13880, Thermo Scientific) was cultured on Nutrition broth (Oxoid, CM0001) overnight (approximately 12 h) at 26°C, 200 rpm. The liquid culture was centrifuged at 10,000 × *g* for 2 min at 4°C to remove residues of the culture medium, and the pellet was washed twice using 1× Phosphate Buffered Saline (PBS, Sigma). The bacterial cells were resuspended in sterile PBS and the solution diluted to achieve an optical density (OD_{600}) of 0.025. Injections were performed on two-day old flies that were fed choice diet prior to the bacterial challenge. A group of 15–20 flies were cold anesthetised at −20°C for 2 m and kept on dry bath (Product code: MK20) at −10°C during the injection. Bacterial cells were injected in the fly at the coxa of the third right leg using a MP4 microinjection system (World Precision Instruments). The injection volume was 0.2 μL dispensed at the rate of 50 nL/s, corresponding to approximately 2,000 bacterial cells/fly. PBS-injected flies were used as controls for injury. After injection, flies were kept in a 1.5 L plastic cage and fed a choice diet. Survival was recorded for six days post-infection (PI). We ran three replicates for each diet treatment (18–22 flies/replicate).

Statistical Analysis

Generalised Linear Models (GLM) with binomial distribution and *quasi* extension were fitted to test for effects of diet manipulation in mothers/fathers/both parents on egg hatching percentage, pupation percentage, emergence percentage, and percentage of larval body lipid reserves. A GLM with Gaussian distribution was used to assess for the effect of diet manipulation in mothers/fathers/both parents on pupal weight and larval weight. Effects of maternal or paternal or parental diet, offspring sex, offspring infection treatment and their interactions on the survival of adult offspring were tested using Cox regression analysis. All analyses were performed in R (R Development Core Team, 2011). Graphs and Kaplan–Meier survival curves were generated from BM SPSS Statistics 25.0.

RESULTS

Effects of Maternal Diet on Offspring Development and Pathogen Resistance

Maternal diet significantly influenced egg hatching percentage (GLM, $F_{4,26} = 9.401$, $P < 0.001$) (**Supplementary Table 1**).

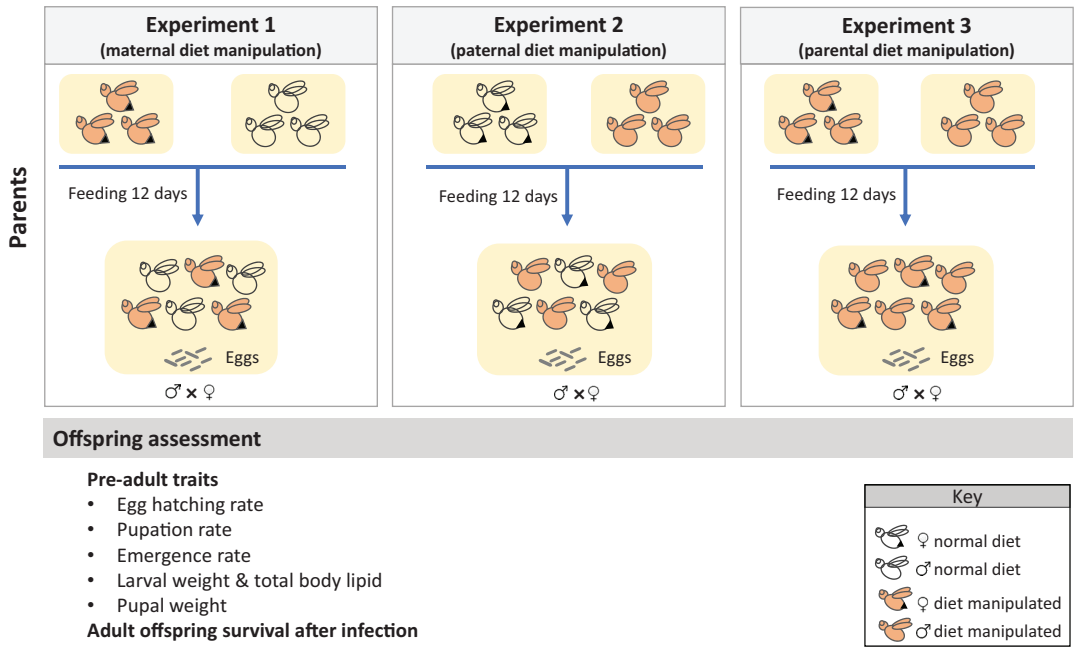

FIGURE 1 | Experimental set up of the three experiments of this study. Effects of maternal, paternal and parental manipulation of the diet were measured on several pre-adult and adult traits of the offspring.

Eggs from mothers fed the protein-biased diet (PC 1:1) had a lower hatching percentage compared to those from the other diet treatments (**Figure 2**). There was no significant effect of the maternal diet on pupation percentage (GLM, $F_{4,24} = 1.964$, $P = 0.132$) and emergence percentage (GLM, $F_{4,24} = 2.570$, $P = 0.064$) (**Supplementary Table 1**). Moreover, maternal diet had no effect on larval weight (GLM, $F_{4,296} = 1.742$, $P = 0.141$), pupal weight (GLM, $F_{4,298} = 1.549$, $P = 0.188$), and percentage of total body lipid in offspring larvae (GLM, $F_{4,90} = 0.726$, $P = 0.576$) (**Supplementary Table 1**).

When survival was analysed during the six first days using a COX regression, we could not detect any statistically significance of the maternal diet treatment on the survival of infected offspring (**Supplementary Table 2**). However and as expected, non-infected offspring survived at a greater rate than infected ones (**Supplementary Table 2**). At day 4, there was in trend where the percentage of dead sons was greater on the two extreme diets (P:C = 1:8 & 1:1), while it was lower when mums where fed the choice diet (**Figure 3A**). We did not observe a similar trend for daughters (**Figure 3B**).

Effects of Paternal Diet on Offspring Development and Pathogen Resistance

Paternal diet did not affect hatching percentage (GLM, $F_{4,40} = 0.852$, $P = 0.500$), pupation percentage (GLM, $F_{4,40} = 0.328$, $P = 0.856$) or emergence percentage (GLM, $F_{4,40} = 0.329$, $P = 0.857$) (**Supplementary Table 3**). We also did not observe any significant effects of paternal diet on larval weight (GLM, $F_{4,297} = 1.575$, $P = 0.181$), pupal weight (GLM, $F_{4,294} = 2.091$, $P = 0.082$), and percentage of total body lipid in offspring larvae (GLM, $F_{4,86} = 0.999$, $P = 0.412$) (**Supplementary Table 3**).

We did not detect any statistically significance of the paternal diet treatment on the survival trajectories of infected offspring (**Supplementary Table 4** and **Figure 4**).

Effects of Parental Diet on Offspring Development and Pathogen Resistance

Parental diet had no effect on hatching percentage (GLM, $F_{4,34} = 0.749$, $P = 0.566$), pupation percentage (GLM, $F_{4,34} = 1.882$, $P = 0.136$), and emergence percentage (GLM, $F_{4,34} = 2.117$, $P = 0.100$) (**Supplementary Table 5**). We also did not observe any significant effects of parental diet on larval weight (GLM, $F_{4,294} = 0.964$, $P = 0.428$), pupal weight (GLM, $F_{4,294} = 2.091$, $P = 0.082$), and percentage of total body lipid in offspring larvae (GLM, $F_{4,99} = 0.706$, $P = 0.589$) (**Supplementary Table 5**).

The interaction between parental diet, offspring sex and infection treatment significantly affected the survival of adult offspring (**Supplementary Table 6**). To better understand the result, we ran four models to test for the effect of parental diet on the survival rate of non-infected males, non-infected females, infected males, and infected females. We found that parental diet significantly affected the survival of infected sons but not infected daughters (**Figure 5**). Particularly, the survival of sons from parents fed PC 1:8, 1:5 and 1:1 were greater than sons from parents fed the PC 1:3 and choice diet (**Figure 5A**). Survival of non-infected sons and daughters was not significantly influenced by parental diet manipulation with, on average, 10% of

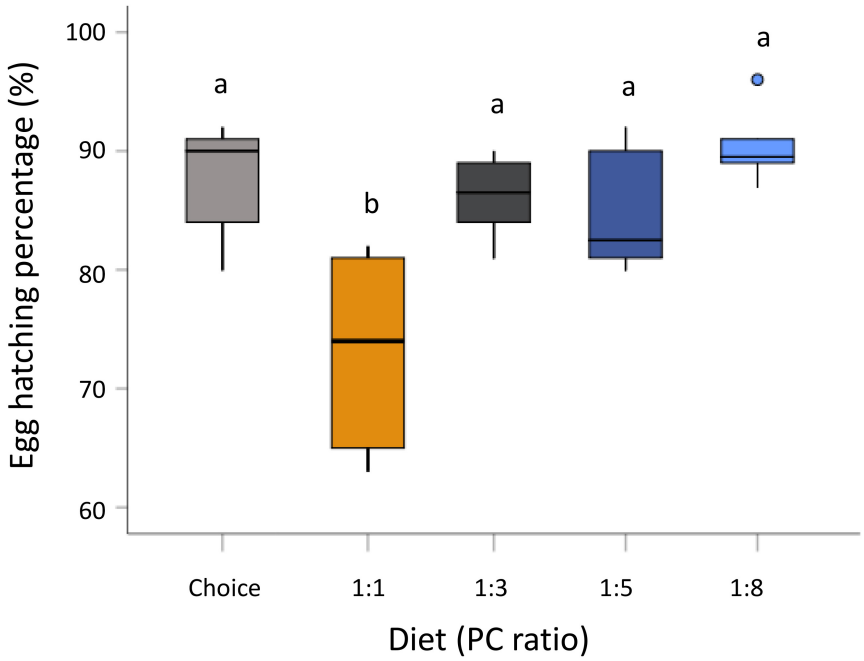

FIGURE 2 | Effect of maternal diet on hatching percentage. Mothers were fed five diets varying in the protein-to-carbohydrate ratio (PC ratio) and egg hatching rate was measured. Bars indicate the percentage of egg hatching. Letters above bars indicate significant difference of egg hatching percentage between diet treatments. Significance was accessed by Student-Newman-Keuls (SNK) test and was considered at $P < 0.05$.

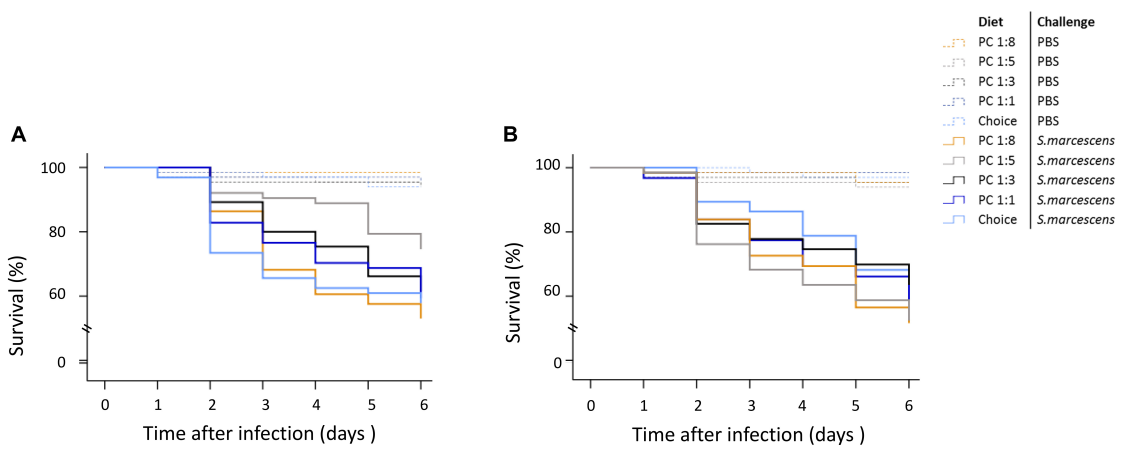

FIGURE 3 | Effect of maternal diet on offspring survival during the first 6 days post septic infection. Mothers were fed one of five diets varying in the protein-to-carbohydrate ratio (PC ratio) whereas fathers were fed a choice diet. Their sons **(A)** and daughters **(B)** were injected with either PBS or *Serratia marcescens* at adult stage. Lines indicates Kaplan–Meier survival curves [dash lines (non-infected flies), continuous lines (infected flies)]. Colours indicate maternal diet treatments [orange (PC 1:1), light grey (choice diet), dark grey (PC 1:3), dark blue (PC 1:5) and light blue (PC 1:8)]. Significant differences between survival curves were determined by Cox regression analysis at $P < 0.05$.

non-infected offspring dying during the 6 first days post infection (**Figure 5**).

DISCUSSION

In this study, we investigated how parental diet (i.e., diet of mothers, fathers, or both) influences offspring developmental traits and adult survival after septic infection with the pathogenic bacterium *S. marcescens*. Surprisingly, when the diet composition was manipulated for both parents, we found that infected sons from parents fed unbalanced diets survived at a greater rate after infection compared to those from parents fed balanced diets. Parental diet manipulation hardly affected offspring developmental traits, with only a lower hatching percentage observed when mothers were fed a protein-biased

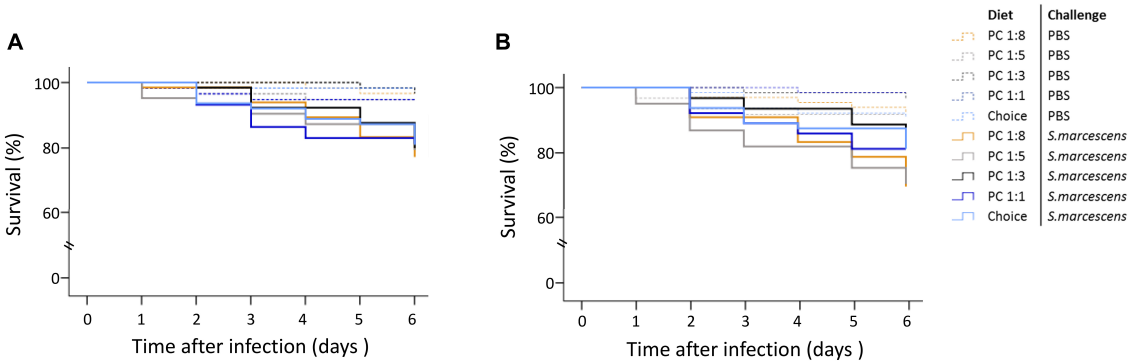

FIGURE 4 | Effect of paternal diet on offspring survival during the first 6 days post septic infection. Fathers were fed one of five diets varying in the protein-to-carbohydrate ratio (PC ratio) whereas mothers were fed a choice diet. Their sons **(A)** and daughters **(B)** were injected with either PBS or *Serratia marcescens* at adult stage. Lines indicates Kaplan–Meier survival curves [dash lines (non-infected flies), continuous lines (infected flies). Colours indicate paternal diet treatments [orange (PC 1:1), light grey (choice diet), dark grey (PC 1:3), dark blue (PC 1:5) and light blue (PC 1:8)]. Significant differences between survival curves were determined by Cox regression analysis at $P < 0.05$.

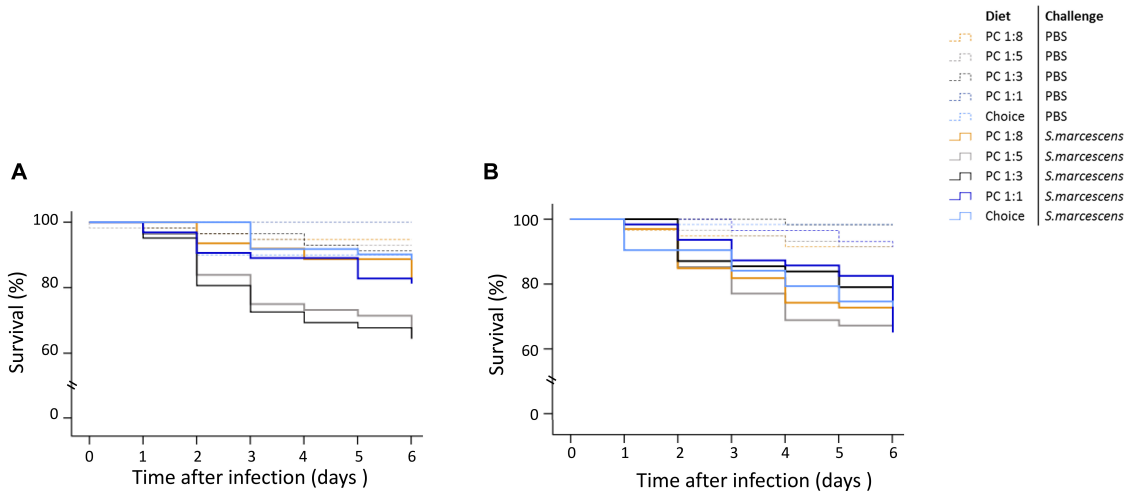

FIGURE 5 | Effect of parental diet on offspring survival during the first 6 days post septic infection. Both mother and fathers were fed one of five diets varying in the protein-to-carbohydrate ratio (PC ratio). Their sons **(A)** and daughters **(B)** were injected with either PBS or *Serratia marcescens* at adult stage. Lines indicates Kaplan–Meier survival curves [dash lines (non-infected flies), continuous lines (infected flies). Colours indicate parental diet treatments [orange (PC 1:1), light grey (choice diet), dark grey (PC 1:3), dark blue (PC 1:5) and light blue (PC 1:8)]. Significant differences between survival curves were determined by Cox regression analysis and was determined at $P < 0.05$.

diet. Paternal and maternal diets did not affect the survival of infected offspring.

A recent study in *Drosophila* has shown that maternal environmental condition other than infection (i.e., cohabitation with a parasitic wasp) results in offspring that are more capable of resisting an infection (Bozler et al., 2020). Our results showed a sex-dependent effects of both parents', not only mothers', diet on offspring pathogen resistance. Previous studies have shown that the parental diet can affect differently the age and size at maturity of male and female offspring (Zizzari et al., 2016). Considering the relationship between hosts' age and body size with resistance to infection (Garbutt and Little, 2017; Soumya et al., 2017), a sex-dependent effect of parental diet on offspring disease resistance is therefore possible. While our knowledge of the mechanisms involved in sex−specific effects are still limited, this might involve sex-specific transfer of genetic material. This is supported by a study in *Drosophila* showing specific transfer of ribosomal DNA from fathers to daughters but not to sons when fathers were fed a protein-rich diet (Aldrich and Maggert, 2015). More investigations of the molecular mechanisms will give us a better understanding of how parental diet can have sex-specific effects.

Transgenerational effects can be adaptive with offspring having greater fitness in environments similar to those experienced by their parents (see for instance, Gluckman and Hanson, 2004; Galloway and Etterson, 2007; Gluckman et al., 2007; Marshall and Uller, 2007; Raubenheimer et al., 2012; Bateson et al., 2014; Burgess and Marshall, 2014;

Murren et al., 2015). Our results showed, however, that offspring from parents fed unbalanced diets had a greater survival despite the mismatch between the nutritional conditions of parents and offspring (i.e., parents were fed unbalanced diets and offspring were fed a balanced diet). Despite this, it remains to be tested whether the same effect is observed when parents are fed a balanced diet and offspring unbalanced diets. We can speculate, in this case, that parents in poor nutritional conditions might have primed their offspring to better survive the infection (see also (Mitchell and Read, 2005; Ben-Ami et al., 2009; Boots and Roberts, 2012). The differences between maternal and paternal environments might be another important factor when explaining transgenerational plasticity. If the environmental conditions experienced by mothers and fathers are different, this may cause a conflict in the parental strategies to maximise their offspring performance. On the other hand, when both parents experienced the same condition, they may transfer the same environmental information to their offspring. In this study, we found sex-specific transgenerational effects when diets of both parents were manipulated but no effects were detected when only the paternal or maternal diet was manipulated. This reflexes the complexity of the effects of parental diet on offspring pathogen resistance that cannot be predicted by measuring the single effects of paternal and maternal diet (see also Valtonen et al., 2012). Further, this study was focused on better understanding the potential eco-evolutionary effects of parental diet, and therefore mating experiments were conducted in large groups rather than in single pairs, which precludes us to infer the contribution of individual variation. The extent to which individual variation vs population level effects contribute to the findings presented here is beyond the scope of this paper and remains an important avenue for future research.

Our study, together with previous work in *Galleria mellonella* moth and *D. melanogaster*, showed no effect of paternal diet on the survival of offspring during infection (Valtonen et al., 2012; Kangassalo et al., 2015). Although effects of poor paternal diet on offspring phenoloxidase activity and expression level of immune genes have been observed in *P. interpunctella* moths (Triggs and Knell, 2012) and *D. melanogaster* (Zajitschek et al., 2017), changes in immune components does not necessarily lead to changes in survival after infection (Adamo, 2004). Measuring different immune traits in offspring in addition to their survival rate would help to gain a more comphrehensive view on how parental diet affects immunity and resistance in offspring. Furthermore, successful host defence involves resistance (i.e., clear pathogen) and/or tolerance (i.e., reduce the damage of the infection on its health) mechanisms (Ayres and Schneider, 2009; Kutzer and Armitage, 2016; Miller and Cotter, 2018); and effects of an infection on can be pathogen-specific. For instance, in *D. melanogaster*, while *Salmonella typhimurium* infection involves resistance mechanism, *Listeria monocytogenes* infection involves tolerance mechanism (Ayres and Schneider, 2009). Given that parental diet can influence both tolerance and resistance in offspring, measuring the bacterial load and fitness traits (e.g., fecundity and growth) of infected offspring would provide helpful information of the mechanisms underlying the effect of parental diet on offspring's survival after infection.

Lastly, we found that parental diet hardly affected offspring developmental traits. These findings are consistent with previous observations in a neriid flies, *Telostylinus angusticollis* showing that only the egg hatching success was affected by maternal diet (Bonduriansky et al., 2016). The weak effects of parental diet on developmental traits might be due to the abundant resources that were available to the offspring at larval stage. Indeed, parental effects on offspring performance have been suggested to be more pronounced when juveniles encounter poor environmental conditions (Marshall et al., 2006; Bonduriansky and Head, 2007; Donelson et al., 2009; Vijendravarma et al., 2010). Interestingly, in *D. melanogaster*, parental diet has weak effects on offspring's phenotype, but was found to significantly influence grand-offspring (Deas et al., 2019). Future investigations measuring the effects of parental diet on offspring traits under different larval dietary conditions and throughout several generations might improve our understanding of the transgenerational effects of nutrition on offspring life-history traits.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

HD and FP designed the experiment. HD and BN collected the data. HD, JM, and FP analysed the data. All authors contributed to the writing and revision of the manuscript, and approved the final version submitted to the journal.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.606993/full#supplementary-material

REFERENCES

Adamo, S. A. (2004). How should behavioural ecologists interpret measurements of immunity? *Anim. Behav.* 68, 1443–1449. doi: 10.1016/j.anbehav.2004.05.005

Aldrich, J. C., and Maggert, K. A. (2015). Transgenerational inheritance of diet-induced genome rearrangements in Drosophila. *PLoS Genet.* 11:e1005148. doi: 10.1371/journal.pgen.1005148

Ayres, J. S., and Schneider, D. S. (2009). The role of anorexia in resistance and tolerance to infections in Drosophila. *PLoS Biol.* 7:e1000150. doi: 10.1371/journal.pbio.1000150

Bateson, P., Gluckman, P., and Hanson, M. (2014). The biology of developmental plasticity and the Predictive Adaptive Response hypothesis. *J. Physiol.* 592, 2357–2368. doi: 10.1113/jphysiol.2014.271460

Ben-Ami, F., Ebert, D., and Regoes, R. R. (2009). Pathogen dose infectivity curves as a method to analyze the distribution of host susceptibility: A quantitative assessment of maternal effects after food stress and pathogen exposure. *Am. Nat.* 175, 106–115. doi: 10.1086/648672

Bonduriansky, R., and Day, T. (2009). Nongenetic inheritance and its evolutionary implications. *Annu. Rev. Ecol. Evol. Syst.* 40, 103–125. doi: 10.1146/annurev.ecolsys.39.110707.173441

Bonduriansky, R., and Head, M. (2007). Maternal and paternal condition effects on offspring phenotype in Telostylinus angusticollis (Diptera: Neriidae). *J. Evol. Biol.* 20, 2379–2388. doi: 10.1111/j.1420-9101.2007.01419.x

Bonduriansky, R., Runagall-McNaull, A., and Crean, A. J. (2016). The nutritional geometry of parental effects: maternal and paternal macronutrient consumption and offspring phenotype in a neriid fly. *Funct. Ecol.* 30, 1675–1686. doi: 10.1111/1365-2435.12643

Boots, M., and Roberts, K. E. (2012). Maternal effects in disease resistance: Poor maternal environment increases offspring resistance to an insect virus. *Proc. R. Soc. B Biol. Sci.* 279, 4009–4014. doi: 10.1098/rspb.2012.1073

Bozler, J., Kacsoh, B. Z., and Bosco, G. (2020). Maternal priming of offspring immune system in Drosophila. *G3 Genes Genomes Genetics* 10, 165–175. doi: 10.1534/g3.119.400852

Brookheart, R. T., and Duncan, J. G. (2016). Drosophila melanogaster: An emerging model of transgenerational effects of maternal obesity. *Mol. Cell. Endocrinol.* 435, 20–28. doi: 10.1016/j.mce.2015.12.003

Buescher, J. L., Musselman, L. P., Wilson, C. A., Lang, T., Keleher, M., Baranski, T. J., et al. (2013). Evidence for transgenerational metabolic programming in Drosophila. *DMM Dis. Model. Mech.* 6, 1123–1132. doi: 10.1242/dmm.011924

Burgess, S. C., and Marshall, D. J. (2014). Adaptive parental effects: The importance of estimating environmental predictability and offspring fitness appropriately. *Oikos* 123, 769–776. doi: 10.1111/oik.01235

Burton, T., and Metcalfe, N. B. (2014). Can environmental conditions experienced in early life influence future generations? *Proc. R. Soc. B Biol. Sci.* 2014:311. doi: 10.1098/rspb.2014.0311

Clarke, A. R., Powell, K. S., Weldon, C. W., and Taylor, P. W. (2011). The ecology of Bactrocera tryoni (Diptera: Tephritidae): what do we know to assist pest management? *Ann. Appl. Biol.* 158, 26–54. doi: 10.1111/j.1744-7348.2010.00448.x

Deas, J. B., Blondel, L., and Extavour, C. G. (2019). Ancestral and offspring nutrition interact to affect life-history traits in Drosophila melanogaster. *Proc. R. Soc. B Biol. Sci.* 286:488809. doi: 10.1098/rspb.2018.2778

Dew-Budd, K., Jarnigan, J., and Reed, L. K. (2016). Genetic and sex-specific transgenerational effects of a high fat diet in drosophila melanogaster. *PLoS One* 11:e0160857. doi: 10.1371/journal.pone.0160857

Dinh, H., Mendez, V., Tabrizi, S. T., and Ponton, F. (2019). Macronutrients and infection in fruit flies. *Insect Biochem. Mol. Biol.* 110, 98–104. doi: 10.1016/j.ibmb.2019.05.002

Donelson, J. M., Munday, P. L., and McCormick, M. I. (2009). Parental effects on offspring life histories: when are they important? *Biol. Lett.* 5, 262–265. doi: 10.1098/rsbl.2008.0642

Eggert, H., Kurtz, J., and Diddens-de Buhr, M. F. (2014). Different effects of paternal transgenerational immune priming on survival and immunity in step and genetic offspring. *Proc. R. Soc. B Biol. Sci.* 281:2089. doi: 10.1098/rspb.2014.2089

Fanson, B. G., Weldon, Christopher, WTaylor, P. W., Pérez-Staples, D., and Simpson, S. J. (2009). Nutrients, not caloric restriction, extend lifespan in Queensland fruit flies (Bactrocera tryoni). *Aging Cell* 8, 514–523. doi: 10.1111/j.1474-9726.2009.00497.x

Galloway, L. F., and Etterson, J. R. (2007). Transgenerational plasticity is adaptive in the wild. *Science* 318, 1134–1136. doi: 10.1126/science.1148766

Garbutt, J. S., and Little, T. J. (2017). Bigger is better: changes in body size explain a maternal effect of food on offspring disease resistance. *Ecol. Evol.* 7, 1403–1409. doi: 10.1002/ece3.2709

Gluckman, P. D., and Hanson, M. A. (2004). Living with the past: Evolution, development, and patterns of disease. *Science* 2014:1095292. doi: 10.1126/science.1095292

Gluckman, P. D., Hanson, M. A., and Beedle, A. S. (2007). Early life events and their consequences for later disease: A life history and evolutionary perspective. *Am. J. Hum. Biol.* 19, 1–19. doi: 10.1002/ajhb.20590

Hancock, D. L., Hamacek, E. L., Lloyd, A. C., and Elson-Harris, M.M. (2000). *The Distribution and Host Plants of Fruit Flies (Diptera: Tephritidae) in Australia*. Brisbane: DPI Publications.

Kangassalo, K., Valtonen, T. M., Roff, D., Pölkki, M., Dubovskiy, I. M., Sorvari, J., et al. (2015). Intra- and trans-generational effects of larval diet on susceptibility to an entomopathogenic fungus, Beauveria bassiana, in the greater wax moth, Galleria mellonella. *J. Evol. Biol.* 28, 1453–1464. doi: 10.1111/jeb.12666

Kutzer, M. A. M., and Armitage, S. A. O. (2016). Maximising fitness in the face of parasites: a review of host tolerance. *Zoology* 119, 281–289. doi: 10.1016/j.zool.2016.05.011

Marshall, D. J., Cook, C. N., and Emlet, R. B. (2006). Offspring size effects mediate competitive interactions in a colonial marine invertebrate. *Ecology* 87, 214–225. doi: 10.1890/05-0350

Marshall, D. J., and Uller, T. (2007). When is a maternal effect adaptive? *Oikos* 116, 1957–1963. doi: 10.1111/j.2007.0030-1299.16203.x

Matzkin, L. M., Johnson, S., Paight, C., and Markow, T. A. (2013). Preadult Parental diet affects offspring development and metabolism in Drosophila melanogaster. *PLoS One* 8:e59530. doi: 10.1371/journal.pone.0059530

Miller, C. V. L., and Cotter, S. C. (2018). Resistance and tolerance: The role of nutrients on pathogen dynamics and infection outcomes in an insect host. *J. Anim. Ecol.* 87, 500–510. doi: 10.1111/1365-2656.12763

Mitchell, S. E., and Read, A. F. (2005). Poor maternal environment enhances offspring disease resistance in an invertebrate. *Proc. R. Soc. B Biol. Sci.* 272, 2601–2607. doi: 10.1098/rspb.2005.3253

Moadeli, T., Taylor, P. W., and Ponton, F. (2017). High productivity gel diets for rearing of Queensland fruit fly, Bactrocera tryoni. *J. Pest Sci.* 90, 507–520. doi: 10.1007/s10340-016-0813-0

Mousseau, T. A., and Fox, C. W. (1998). The adaptive significance of maternal effects. *Trends Ecol. Evol.* 13, 403–407. doi: 10.1016/S0169-5347(98)01472-4

Murren, C. J., Auld, J. R., Callahan, H., Ghalambor, C. K., Handelsman, C. A., Heskel, M. A., et al. (2015). Constraints on the evolution of phenotypic plasticity: Limits and costs of phenotype and plasticity. *Heredity* 115, 293–301. doi: 10.1038/hdy.2015.8

Öst, A., Lempradl, A., Casas, E., Weigert, M., Tiko, T., Deniz, M., et al. (2014). Paternal diet defines offspring chromatin state and intergenerational obesity. *Cell* 159, 1352–1364. doi: 10.1016/j.cell.2014.11.005

Pigliucci, M. (2001). *Phenotypic Plasticity: Beyond nature and nurture: Syntheses in ecology and evolution*. Baltimore: BaltimoreJohns Hopkins University Press.

Prasad, N. G., Shakarad, M., Rajamani, M., and Joshi, A. (2003). Interaction between the effects of maternal and larval levels of nutrition on pre-adult survival in Drosophila melanogaster. *Evol. Ecol. Res.* 5, 903–911.

Qvarnström, A., and Price, T. D. (2001). Maternal effects, paternal effects and sexual selection. *Trends Ecol. Evol.* 16, 95–100. doi: 10.1016/S0169-5347(00)02063-2

R Development Core Team (2011). *R: A language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Raubenheimer, D., Simpson, S. J., and Tait, A. H. (2012). Match and mismatch: Conservation physiology, nutritional ecology and the timescales of biological adaptation. *Philos. Trans. R. Soc. B Biol. Sci.* 367, 1628–1646. doi: 10.1098/rstb.2012.0007

Roth, O., Joop, G., Eggert, H., Hilbert, J., Daniel, J., Schmid-Hempel, P., et al. (2010). Paternally derived immune priming for offspring in the red flour beetle, Tribolium castaneum. *J. Anim. Ecol.* 79, 403–413. doi: 10.1111/j.1365-2656.2009.01617.x

Saastamoinen, M., Hirai, N., and van Nouhuys, S. (2013). Direct and transgenerational responses to food deprivation during development in the Glanville fritillary butterfly. *Oecologia* 171, 93–104. doi: 10.1007/s00442-012-2412-y

Soumya, K., Ganga Visalakshy, P. N., Krishnamoorthy, A., Kavitha, S. J., and Gopalakrishna Pillai, K. (2017). Host-age-dependent parasitism and reproductive success of *Apanteles stantoni* (Hymenoptera: Braconidae), a larval parasitoid of *Diaphania indica* (Lepidoptera: Pyralidae). *Bio. Sci. Tech.* 27, 70–80. doi: 10.1080/09583157.2016.1253829

Sutherst, R. W., Collyer, B. S., and Yonow, T. (2000). The vulnerability of australian horticulture to the queensland fruit fly, Bactrocera (Dacus) tryoni, under climate change. *Aust. J. Agric. Res.* 51, 467–480. doi: 10.1071/AR98203

Triggs, A. M., and Knell, R. J. (2012). Parental diet has strong transgenerational effects on offspring immunity. *Funct. Ecol.* 26, 1409–1417. doi: 10.1111/j.1365-2435.2012.02051.x

Uller, T., Nakagawa, S., and English, S. (2013). Weak evidence for anticipatory parental effects in plants and animals. *J. Evol. Biol.* 26, 2161–2170. doi: 10.1111/jeb.12212

Valtonen, T. M., Kangassalo, K., Pölkki, M., and Rantala, M. J. (2012). Transgenerational effects of parental larval diet on offspring development time, adult body size and pathogen resistance in Drosophila melanogaster. *PLoS One* 7:0031611. doi: 10.1371/journal.pone.0031611

Vijendravarma, R. K., Narasimha, S., and Kawecki, T. J. (2010). Effects of parental larval diet on egg size and offspring traits in Drosophila. *Biol. Lett.* 6, 238–241. doi: 10.1098/rsbl.2009.0754

West-Eberhard, M. J. (2003). *Developmental Plasticity and Evolution*. New York, NY: Oxford University Press.

Whitman, D., and Agrawal, A. (2009). *What is phenotypic plasticity and why is it important?*. New York,NY: Science Publishers, doi: 10.1201/b10201-2

Woestmann, L., and Saastamoinen, M. (2016). The importance of transgenerational effects in lepidoptera. *Curr. Zool.* 62, 489–499. doi: 10.1093/cz/zow029

Zajitschek, F., Zajitschek, S., and Manier, M. (2017). High-protein paternal diet confers an advantage to sons in sperm competition. *Biol. Lett.* 13:20160914. doi: 10.1098/rsbl.2016.0914

Zanchi, C., Troussard, J. P., Martinaud, G., Moreau, J., and Moret, Y. (2011). Differential expression and costs between maternally and paternally derived immune priming for offspring in an insect. *J. Anim. Ecol.* 80, 1174–1183. doi: 10.1111/j.1365-2656.2011.01872.x

Zirbel, K., Eastmond, B., and Alto, B. W. (2018). Parental and offspring larval diets interact to influence life-history traits and infection with dengue virus in Aedes aegypti. *R. Soc. Open Sci.* 5:180539. doi: 10.1098/rsos.180539

Zizzari, Z. V., van Straalen, N. M., and Ellers, J. (2016). Transgenerational effects of nutrition are different for sons and daughters. *J. Evol. Biol.* 29, 1317–1327. doi: 10.1111/jeb.12872

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