What happens to offspring when parents are inbred, old or had a poor start in life? Evidence for sex-specific parental effects

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Abstract

Parental effects on offspring performance have been attributed to many factors such as parental age, size and condition. However, we know little about how these different parental characteristics interact to determine parental effects, or the extent to which their effect on offspring depends on either the sex of the parent or that of the offspring. Here we experimentally tested for effects of variation in parents' early diet and inbreeding levels, as well as effects of parental age, and for potential interactive effects of these three factors on key aspects of offspring development in the mosquitofish (*Gambusia holbrooki*). Older mothers produced offspring that were significantly smaller at birth. This negative effect of maternal age on offspring size was still evident at maturation as older mothers had smaller daughters, but not smaller sons. The daughters of older mothers did, however, reach maturity sooner. Paternal age did not affect offspring body size, but it had a complex effect on their sons' relative genital size. When initially raised on a food-restricted diet, older fathers sired sons with relatively smaller genitalia, but when fathers were initially raised on a control diet their sons had relatively larger genitalia. The inbreeding status of mothers and fathers had no significant effects on any of the measured offspring traits. Our results indicate that the manifestation of parental effects can be complex. It can vary with both parent and offspring sex; can change over an offspring's life; and is sometimes evident as an interaction between different parental traits. Understanding this complexity will be important to predict the role of parental effects in adaptation.

Introduction

There are multiple ways in which parents can influence the phenotype of their offspring (Kirkpatrick & Lande, 1989; Uller, 2008; Bonduriansky & Day, 2009). The best-known pathway is that offspring inherit genes that cause them to resemble their parents (i.e. due to additive genetic effects). However, parental phenotype and genotype can also influence their offsprings' phenotype via other routes (Mousseau & Fox, 1998; Räsänen & Kruuk, 2007; Wolf & Wade, 2009). For example, the

environmental conditions that a parent experiences (such as diet or disease), parental age, parental body condition and nonadditive genetic effects (e.g. parental heterozygosity) can all shape a parent's phenotype and hence can determine 'parental effects' on offspring (e.g. Annavi *et al.*, 2014; Bouwhuis *et al.*, 2015; Besson *et al.*, 2016). Variation in parental effects can have substantial implications for offspring fitness that are often equivalent in magnitude to those arising from heritable genetic variation, with ramifications for both evolutionary and ecological dynamics (Lynch & Walsh, 1998; Räsänen & Kruuk, 2007; Badyaev & Uller, 2009; Bonduriansky & Day, 2018). The complexity of parental effects is becoming increasingly apparent: identifying the multiple drivers of changes in parental phenotype that then affect factors transferred to offspring, and determining when, and how, these drivers interact, is

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an ongoing challenge for evolutionary ecologists (Mousseau & Fox, 1998; Uller, 2008; Royle et al., 2012; Bonduriansky & Day, 2018).

It is well known that parental effects can alter offspring morphology, growth, development and behaviour (Mousseau & Fox, 1998; Jonsson & Jonsson, 2014). There are, however, many details that remain unresolved, partly because we rarely understand the proximate mechanisms that underpin parental effects. First, there is far more evidence for maternal than paternal effects (Curley et al., 2011; Crean et al., 2013). Second, we still know surprisingly little about the direction and relative magnitude of parental effects associated with specific parental phenotypes. Third, there is almost no understanding of how different parental factors might interact to shape offspring's phenotype. Four, few studies have investigated the extent to which parental effects differ for sons and daughters (but see, e.g. Pembrey et al., 2006; Kruuk et al., 2015).

To date, three particular parental traits have drawn attention for their likely effects on offspring, namely parental age, parental body condition and parental inbreeding status. First, parental age has been shown to have predictable effects (e.g. Bouwhuis et al., 2015; Schroeder et al., 2015). For example, there is a trend for older mothers to produce shorter-lived offspring (the 'Lansing effect'; Lansing, 1947; Hercus & Hoffmann, 2000; Priest et al., 2002); and in insects, for example, egg hatching rates and larval viability decline with maternal age (Hercus & Hoffmann, 2000; Fox et al., 2003; Singh & Omkar, 2009). Second, parental condition affects offspring phenotype in many species (review: Bonduriansky & Day, 2009; Bonduriansky et al., 2018). This effect is most obvious when parents provide food to their offspring (Smiseth et al., 2012). For example, decreased parental provisioning, due to poorer parental body condition, negatively affects offspring body size and horn length in dung beetles (Hunt & Simmons, 2000). Similarly, maternal nutrition often affects offspring phenotype through its effect on the allocation of resources into eggs (Bernardo, 1996a; Mousseau & Fox, 1998). Third, inbreeding negatively affects many adult traits ('inbreeding depression'; Keller & Waller, 2002). There is, for example, evidence that more inbred parents produce offspring with lower hatching rates (Mattey et al., 2013; Pooley et al., 2014), reduced body weight (Bérénos et al., 2016), decreased juvenile survival (Huisman et al., 2016), weaker immune responses (Reid et al., 2003) and lower reproductive success (Szulkin et al., 2007), although some studies find no evidence that inbred parents produce less fit offspring (Keller et al., 2002). This suggests that the effect of parental inbreeding on offspring phenotype might vary across taxa.

Despite increasing evidence for the importance of the three well-studied parental characteristics of age, condition and inbreeding status in determining the direction of parental effects, we still lack answers to three key questions. First, we do not know the extent to which these three factors interact to affect the parental phenotype and how this, in turn, maps onto the transfer of nongenetic factors to offspring to determine their phenotype. For example, are detrimental parental effects due to inbreeding status heightened in older parents, or in parents in poor body condition? Answering such questions could provide valuable insights into the proximate mechanisms that generate parental effects. Second, we lack information about the extent to which the parental effect of these three well-studied traits differ for sons and daughters (but see Naguib & Gil, 2005; Walzer & Schausberger, 2013). What makes a high-quality parent might vary depending on the developmental requirements of offspring of each sex (Kruuk et al., 2015). Mothers that are small due to food limitation, for example, might produce small daughters, but standard-sized sons (e.g. Walzer & Schausberger, 2013). This could be due to adaptive shifts in parental allocation or nonadaptive effects arising from physiological differences in how resources affect offspring development in each sex. It is possible that there are complex interactions between genetic and nongenetic inheritance. For example, if a parent transmits genes for slower than average growth to its offspring and the fitness consequences of this differ for sons and daughters due to intra-locus sexual conflict, this might select for adaptive, sex-specific shifts in parental investment that affect offspring growth. In general, we still know little about whether parental age, inbreeding status and body condition, predictably differ in how they affect sons and daughters. Third, it is also still unclear to what extent the effects of these three factors depend on the sex of the parent, thereby creating differences in any resultant maternal and paternal effects.

In species without post-natal parental care, there is far more evidence for maternal than paternal effects (Bernardo, 1996b; Soubry et al., 2014). Maternal provisioning of eggs with chemicals and nutrients offers a straightforward route whereby mothers can affect early offspring development (e.g. Räsänen & Kruuk, 2007; Stynoski et al., 2014). Indeed, variation in the protein and RNA content of eggs is known to directly affect early gene expression in offspring (Fox & Czesak, 2000; Johnstone & Lasko, 2001; Ducatez et al., 2012). There is also evidence in nonmammalian live-bearing species without post-natal care that mothers can provide nutrients such as calcium in the prenatal stages (e.g. Stewart et al. 2010). In contrast, it is less clear how paternal effects arise in the absence of male parental care: sperm is mainly considered to be a device to transfer DNA to eggs (Karr et al., 2009; Crean & Bonduriansky, 2014). Even so, there is increasing evidence that fathers can affect the phenotype of their offspring via nongenetic factors, even in the absence of direct parental care (e.g. Bonduriansky & Head, 2007; Mashoodh et al., 2012;

Crean & Bonduriansky, 2014; Zajitschek *et al.*, 2014; Fay *et al.*, 2016). For example, a father's diet influences offspring size and age at maturity in springtails (Zizzari *et al.*, 2016). This could be due to the presence of non-genetic factors in ejaculates, such as lipids and proteins that enter the zygote; or to epigenetic changes in paternal DNA that then affect gene expression in zygotes (Crean & Bonduriansky, 2014; Holman & Price, 2014). Mothers can also differentially allocate resources to offspring based on their mate's phenotype (e.g. Gil *et al.*, 1999). Although mediated by the mother, such differential allocation is ultimately attributable to the male's phenotype, so it can also be described as a paternal effect (Sheldon, 2000). To date, however, studies that directly compare the relative magnitude of maternal and paternal effects are rare, particularly in taxa lacking post-natal parental care.

Here we tested how three key parental traits in the eastern mosquitofish (*Gambusia holbrooki*): their age, their early development and their inbreeding status affect their offspring's phenotype. We know that all three factors can lead to changes in offspring phenotype in other species (e.g. age: Hercus & Hoffmann, 2000; diet: Bonduriansky & Head, 2007; inbreeding: Mattey *et al.*, 2013), but it is unknown how general these patterns are and how these factors interact to effect offspring phenotype. Previous work on *G. holbrooki* indicates there is substantial variation among mothers in maternal effects (Kruuk *et al.*, 2015; Vega-Trejo *et al.*, 2018) and that there is an effect of inbreeding on male reproductive success (Head *et al.*, 2017; Vega-Trejo *et al.*, 2017). Here, we investigate the relative role of the three focal factors in generating both maternal and paternal effects and quantify their relative importance for sons and daughters.

Materials and methods

Study species

The eastern mosquitofish (*Gambusia holbrooki*) is a poeciliid fish endemic to North America, but now found worldwide (Pyke, 2005). It was introduced to Australia in 1925 (Lloyd, 1986). Although heterozygosity levels are assumed to be lower in Australia than in North America (Ayres *et al.*, 2010), our study population shows a level of heterozygosity (mean heterozygosity: 0.27; Head *et al.*, 2017) that is within the range seen in native populations (0.23–0.63; Vera *et al.*, 2016). *Gambusia* have internal fertilization: females provision eggs prior to fertilization and subsequently provide no further contribution to their offspring (i.e. lecithotrophy; Fernández-Delgado & Rossomanno, 1997). Males mate using coercive 'sneak' copulations in which they approach a female from behind, thrust their gonopodium towards her gonopore (Bisazza & Marin, 1995; Langerhans, 2011) and transfer sperm to the female via a modified anal fin called the gonopodium (Constantz, 1989). Body size in both sexes has been linked to reproductive success. Smaller males show greater manoeuvrability, which seems to increase their success at sneak copulations (Pilastro *et al.*, 1997; Head *et al.*, 2017; but see Booksmythe *et al.* 2016), although larger males can dominate their rivals for access to females and might transfer more sperm because they have larger sperm reserves (Bisazza & Marin, 1991; O'Dea *et al.*, 2014). Female body size is strongly positively correlated with fecundity (Bisazza *et al.*, 1989; Callander *et al.*, 2012). Time to maturation is highly variable, ranging from 25 to 120 days in the laboratory (Livingston *et al.*, 2014; Vega-Trejo *et al.*, 2016a). Mosquitofish rarely live longer than 12–15 months in the wild, but can survive up to 18 months in captivity (Pyke, 2008). Finally, heterozygosity has been shown to be positively correlated with male reproductive success, based on both standing variation and experimental manipulation of inbreeding status (Head *et al.*, 2017; Vega-Trejo *et al.*, 2017).

Experimental design

We tested for the impact of parental age, early diet and inbreeding status on maternal and paternal effects in two separate experiments. *Maternal* effects were investigated by mating experimental females to random stock males, and *paternal* effects by mating experimental males to random stock females (see details below). Stock individuals used in this part of our experiment were laboratory reared offspring originating from pregnant females caught in the ponds as the original stock.

Parental breeding design and fish rearing

Our experimental design consisted of parents who were either inbred or outbred, and then reared on either a control or restricted diet. Our starting population (generation F_0) consisted of offspring from 151 gravid wild-caught females collected from three sites around Canberra, Australia (Lake Ginninderra: 35.228°S, 149.063°E, Lake Burley Griffin: 35.289°S, 149.099°E, and Bruce Ponds: 35.241°S, 149.091°E), from October 2009 to April 2010. As soon as fish were able to be sexed, we separated males and females. We considered females to be mature when yellow spots were evident in the abdomen, indicating yolked eggs (Stearns, 1983). We considered males to be mature when their gonopodium was translucent, with a spine visible at the tip (Stearns, 1983; Zulian *et al.*, 1993). These fish were kept in single-sexed tanks (30–60 fish per 90-L tank) under a 14 : 10 photoperiod in temperature-controlled rooms at 28 °C and fed *ad libitum* with *Artemia* nauplii and commercial fish flakes. We then paired males and females randomly from this starting population to

create 58 full-sib families (F_1; Fig. 1). Fish from these full-sib families were mated to create an F_2 generation of both inbred (inbreeding coefficient $f = 0.25$; corroborated by a 23% reduction in genomewide heterozygosity based on SNPs; Vega-Trejo *et al.*, 2017) and outbred offspring (Fig. 1). To do this, we used pairs of full-sib families (e.g. family A and family B in block 1, where 'block' represents a pair of families) and created outbred offspring by pairing across families (i.e. female from A and male from B, and male from A and female from B), and inbred offspring by pairing within families (i.e. female and male from A, female and male from B). One generation of inbreeding is sufficient to significantly reduce the ability of males to gain paternity, indicative of inbreeding depression (Marsh *et al.*, 2017; Vega-Trejo *et al.*, 2017).

We raised a maximum of 10 fish per cross-type individually in 1-L tanks that were allocated randomly to shelves in a temperature-controlled room under a 14 : 10 photoperiod at 28 °C. Individuals from each brood were evenly split between the food treatments: either a control or restricted (low) food diet (see Vega-Trejo *et al.*, 2016a; Fig. 1). Fish on the control diet were fed *ad libitum* with *Artemia* nauplii twice a day from birth until the end of the experiment. Fish on the low-food diet were fed the control diet until they were 1 week old, and were then fed 3 mg of *Artemia* nauplii once every other day (i.e. approximately < 25% of the control food intake) for 21 days during which they barely grow (see Livingston *et al.*, 2014). We returned them to the control diet after 21 days, so that all fish were on the control diet when they were used as parents. An effect of dietary treatment would therefore indicate that parental juvenile development influences the magnitude of parental effects. The parents in our experiment were F_2 fish
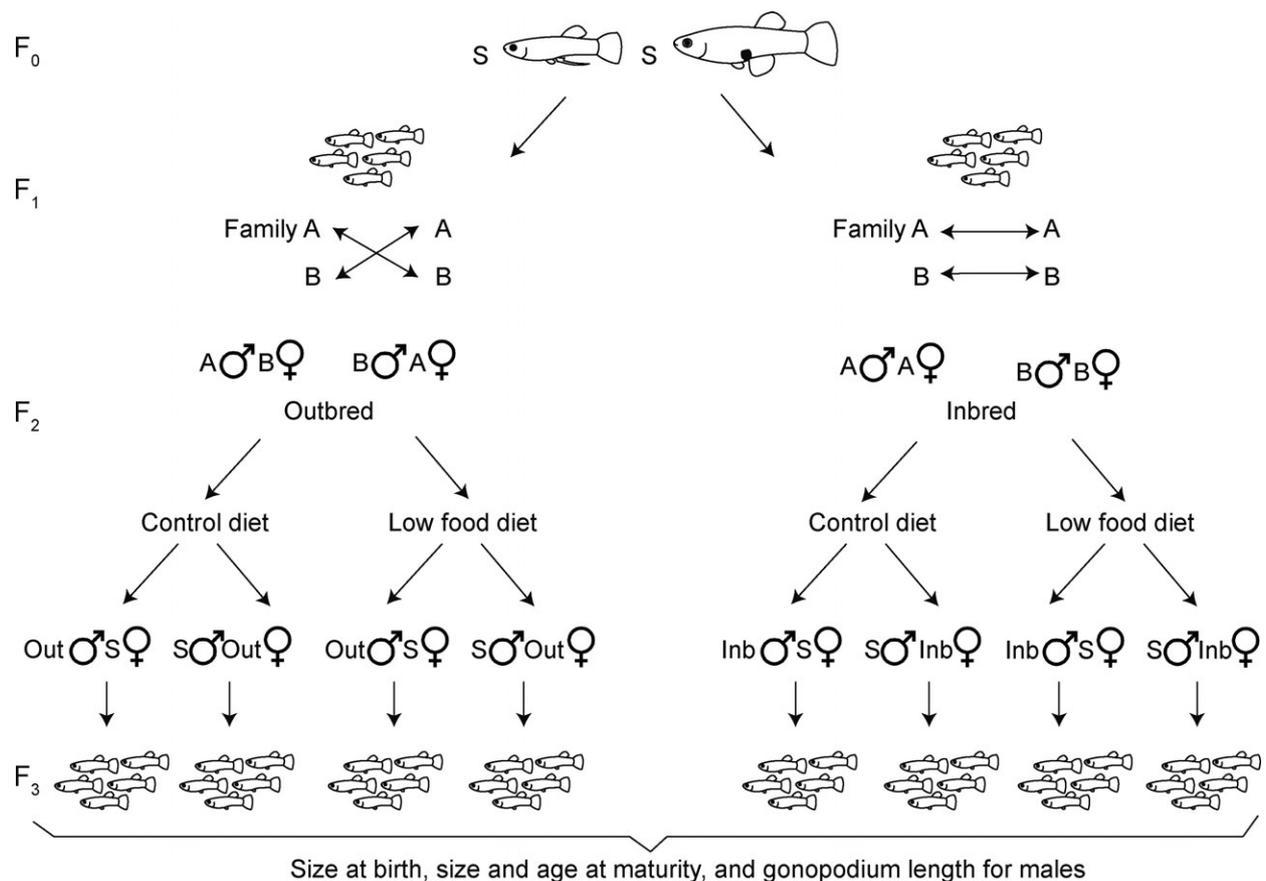

Fig. 1 Schematic of the experimental design. Stock 1 = stock fish. S = stock fish unrelated to stock fish 1. F_0 stock males and females were paired to create F_1 full-sib families (e.g. A and B). We set up 1–4 F_1 females (to maximize the number of offspring) per cross-type to create F_2 outbred (AB, BA; Out) and inbred (AA, BB; Inb) fish. These fish were reared on either a control or a low-food diet early in life. F_2 females from each treatment were paired with a stock (outbred) male to create F_3 offspring on which traits were measured. F_2 males from each treatment artificially inseminated stock (outbred) females to create F_3 offspring on which traits were measured. F_2 and F_3 fish were raised individually.

that survived until maturity and developed normally (e.g. no spinal curvature, which was unrelated to inbreeding depression, $n = 498$ of 527 F_2 offspring at birth).

Our design resulted in four parental types in the F_2 generation (inbred or outbred, reared on a low or control diet), whom we then bred at different ages by mating them to stock (nonexperimental) individuals. Note that neither inbreeding status nor rearing diet influenced survival to maturity of the F_2 generation, but both males and females matured later when they were initially on a low-food diet (Vega-Trejo *et al.*, 2016a). Given that age at maturation in *G. holbrooki* is highly variable (see Pyke, 2005; Livingston *et al.*, 2014; Vega-Trejo *et al.*, 2016a), we considered two measures of parental age in our analyses: 'chronological age' (days since birth) and 'age since maturity' (days since maturity). The results were qualitatively similar for both measures but were more consistently significant for age since maturity. We therefore only present analyses using parental age since maturity in the main text, but analyses and results using chronological age are provided in the online supplement. All data on offspring were collected blind to parental age, diet and inbreeding status.

Experiment 1: Maternal effects

Virgin F_2 females from each of the four treatment groups were mated to stock males ($n = 94$–99 females per treatment). Each female was placed with a single male in a 6.5-L tank for 1 week to mate. She was then transferred into a separate 1-L tank which we checked twice daily for offspring. Females that did not breed within 6 weeks were re-introduced to their original male for another week. We recorded female age (days from maturity until she gave birth), size (standard length, SL in mm), and how many offspring she produced. Means $\pm$ SD for mother's age since maturity are shown in Table S1. Once females gave birth, we individually raised 1–10 (average 4.3 offspring) F_3 offspring per mother in 1-L tanks. We recorded their size at birth, and size and age at maturity, and (for sons) gonopodium length (for detailed methods see below). All offspring were fed *ad libitum* with *Artemia* nauplii twice daily.

We obtained data for 945 offspring from 37 outbred/control-diet mothers, 38 inbred/control-diet mothers, 47 outbred/low-diet mothers and 42 inbred/low-diet mothers. There were no significant differences among the four types of mothers, or among mothers of different ages in whether or not they produced offspring (generalized linear model with binomial distribution: maternal type: $\chi^2_{(3)} = 3.714$, $P = 0.294$, maternal age: $\chi^2_{(1)} = 0.002$, $P = 0.966$, interaction maternal type $\times$ maternal age: $\chi^2_{(3)} = 3.007$, $P = 0.391$). There was also no difference in the number of offspring produced by

each treatment (linear model: effect of maternal type: $\chi^2_{(3)} = 0.306$).

Experiment 2: Paternal effects

In the paternal effects experiment, we used artificial insemination to control for any confounding effects of differential maternal allocation in response to a male's phenotype. We took sperm from F_2 males from each of the four treatments ($n = 36$ males/treatment) and used it to artificially inseminate two laboratory stock females per male (Fig. 1). To perform the inseminations, we first anaesthetized the male in ice-cold water. We then placed him on a glass slide with his gonopodium swung forward and put 100 μL of saline solution (0.9%NaCl) at the gonopodium tip. We applied gentle pressure to the male's abdomen to expel sperm (Matthews *et al.*, 1997). We then used a micropipette to transfer 10 intact sperm bundles (in 3 μL saline solution) directly into the reproductive tract of each of two anaesthetized females. We recorded male age (days from maturity until he was used to inseminate the females), and how many offspring were produced (42% of artificial inseminations yielded offspring). We used males that ranged from 36 to 171 days post-maturity (mean $\pm$ SD: 124.4 $\pm$ 32.4) and male age was thus treated as a continuous variable. Means $\pm$ SD for male age for each treatment are shown in Table S1. We then placed the inseminated females in individual 1-L tanks and allowed them 6 weeks to give birth, checking for offspring twice daily. We reared a maximum of ten offspring per female in separate 1-L tanks and recorded their size at birth, size and age at maturity, and (for sons) gonopodium length (for detailed methods see below). All offspring were fed *ad libitum* with *Artemia* nauplii twice daily.

We obtained data for 378 offspring sired by 18 outbred/control-diet males, 21 inbred/control-diet males, 27 outbred/low-diet males and 25 inbred/low-diet males. There was no significant difference among the four types of males, or an effect of paternal age, on whether or not males sired any offspring (generalized linear model with binomial distribution: paternal type: $\chi^2_{(3)} = 0.730$ $P = 0.866$, paternal age: $\chi^2_{(1)} = 0.004$, $P = 0.953$, interaction: $\chi^2_{(3)} = 1.708$, $P = 0.635$).

Offspring phenotype measurements

To measure offspring size, all offspring were photographed < 18 h after birth. They were placed in a plastic dish (27 $\times$ 27 mm) with 2 mm depth of water to restrict movement and photographed from above. To measure size at maturity, fish were anaesthetized in ice-cold water for a few seconds to reduce movement then photographed alongside a microscopic ruler (0.1 mm gradation). We used Image J software (Abramoff *et al.*, 2004) to measure standard length

(SL = snout tip to base of caudal fin) for both sexes, and gonopodium length (apical tip to base) for males. To determine offspring maturity, we inspected fish three times a week. We calculated relative gonopodium length for males as the residuals from a linear regression of (log) gonopodium length on (log) standard length. In total, we measured the following traits on offspring of both sexes: (i) size at birth; (ii) size at maturity; (iii) age at maturity; and (iv) males only: relative gonopodium length. All inspections were made blind to treatment.

Statistical analyses

To determine parental effects on offspring traits (size at birth, size at maturity, age at maturity and relative gonopodium length), we ran separate general linear mixed models (GLMM) for each experiment and each trait using the package lme4 (Bates *et al.*, 2015) in R version 3.0.2 (R Development Core Team, 2012). We included parental age (age from maturity to age when the female gave birth or age when the male was used to inseminate a female), parental diet (control or low), inbreeding status (outbred or inbred), sex of the offspring (except for relative gonopodium size) and all possible two-way interactions as fixed effects, and we specified a Gaussian error structure for all traits given the data distributions. For tractability of interpretation, we excluded three-way interactions. Each model was fitted with maternal identity and parental block (i.e. pair of families) as random effects. For the paternal effects analyses, we additionally included paternal identity as a random effect because each male could sire up to two broods (33% of the males successfully inseminated two females). Paternal identity was excluded from the maternal effects models because each mother was paired with only a single stock male. We explicitly excluded parental size as a predictor variable in our model because it is a post-treatment variable (see Gelman & Hill, 2007) which might be affected by the factors of causal interest, namely inbreeding status and parental diet. However, for completeness, we re-ran the analyses including parental size, and the conclusions remained unchanged (see Data S1).

We standardized all continuous variables (both predictors and dependent) to zero mean and unit variance across the entire data set (i.e. across the maternal and paternal experiment, except for relative gonopodium length—which was standardized separately for the maternal and paternal experiments) prior to analyses, to facilitate model convergence and interpretation of the output of models containing interactions. All model terms were tested for significance using the ANOVA function in the *car* package (Fox & Weisberg, 2011) specifying Type III Wald chi-square tests. All nonsignificant interactions were sequentially removed. Only final models are presented.

Results

The means ($\pm$ SE) of the four offspring traits that we measured are given in Table S2 separated by: offspring sex; maternal or paternal diet, and maternal or paternal inbreeding status.

Maternal effects

There were no differences in the number of offspring produced by the four types of mothers or by mothers of different ages (linear model: maternal type: $\chi^2_{(3)} = 2.514$, $P = 0.061$, maternal age: $\chi^2_{(1)} = 2.206$, $P = 0.140$, interaction maternal type $\times$ maternal age: $\chi^2_{(3)} = 2.688$, $P = 0.049$).

Older mothers gave birth to significantly smaller offspring, regardless of whether these were sons or daughters ($P = 0.005$; Table 1, Fig. 2). In contrast, the effect that maternal age had on both offspring size and age *at maturation* differed between sons and daughters (offspring sex $\times$ maternal age interactions: $P = 0.012$ for size at maturation and $P = 0.001$ for age at maturation, respectively; Table 1). Daughters of older mothers were significantly smaller at maturity ($P = 0.014$) and matured significantly earlier ($P < 0.001$), but there were no equivalent effects on sons ($P = 0.760$ and 0.593 for their size and age at maturity, respectively; Fig. 3a,b).

Neither maternal inbreeding status (all $P > 0.459$), diet (all $P > 0.069$), nor their interaction (all $P > 0.079$) had significant effects on offspring traits (Table 1).

Paternal effects

There was a tendency for older fathers to sire offspring that were smaller at birth, but the effect was marginally nonsignificant ($P = 0.054$; Table 2). It was, however, almost identical in magnitude to the estimated effect of maternal age on size at birth (maternal: -0.179 ± 0.064 SE; paternal: -0.179 ± 0.093 SE). There was no evidence for an effect of father's age on offspring size or age at maturation (both $P > 0.275$; Table 2), but there were complex effects on genital size. The effect of a father's age since maturity on his sons' relative gonopodium length depended on his diet (interaction between age and diet; $P = 0.003$; Table 2). Older fathers reared on the low-diet sired sons with a shorter relative gonopodium than did younger males ($P = 0.029$). However, on the control diet, the pattern was the opposite: older fathers sired sons with a longer relative gonopodium than did younger males ($P = 0.039$; Table 2; Fig. 4).

Paternal diet had no effect on offspring size at birth or size at maturity (both $P > 0.241$). However, fathers reared on the low-diet sired offspring that took longer to mature ($P = 0.017$; Table 2).

A father's inbreeding status had no effect on offspring size at birth, or offspring size or age at maturity (all

Response variable	Predictor	Estimate	SE	$\chi^2_{(1)}$	P
Size at birth	Intercept	0.229	0.120	3.651	0.056
(*N* offspring = 868)	Sex (male)	0.058	0.054	1.156	0.282
(*N* Mothers = 226)	Mother's age since maturity	**−0.179**	**0.064**	**7.916**	**0.005**
	Mother's diet (low-food)	−0.034	0.129	0.068	0.794
	Mother's inbreeding status (inbred)	−0.083	0.124	0.454	0.500
Random effects	Mother's ID variance	0.624			
	Block variance	0.040			
	Residual variance	0.714			
Size at maturity	Intercept	0.719	0.085	71.648	<0.001
(*N* offspring = 868)	Sex (male)	**−0.933**	**0.055**	**284.069**	**<0.001**
(*N* Mothers = 226)	Mother's age since maturity	**−0.123**	**0.050**	**6.090**	**0.014**
	Mother's diet (low)	−0.081	0.086	0.897	0.344
	Mother's inbreeding status (inbred)	−0.061	0.082	0.549	0.459
	Sex × Mother's age since maturity	**0.139**	**0.056**	**6.247**	**0.012**
Random effects	Mother's ID variance	0.164			
	Block variance	0.031			
	Residual variance	0.766			
Age at maturity	Intercept	0.622	0.083	55.569	<0.001
(*N* offspring = 858)	Sex (male)	**−0.984**	**0.056**	**305.641**	**<0.001**
(*N* Mothers = 226)	Mother's age since maturity	**−0.167**	**0.050**	**11.181**	**0.001**
	Mother's diet (low)	−0.155	0.085	3.317	0.069
	Mother's inbreeding status (inbred)	−0.021	0.082	0.067	0.797
	Sex × Mother's age since maturity	**0.195**	**0.057**	**11.849**	**0.001**
Random effects	Mother's ID variance	0.155			
	Block variance	0.026			
	Residual variance	0.777			
Relative gonopodium size	Intercept	0.040	0.095	0.172	0.678
(*N* offspring = 418)	Mother's age since maturity	−0.086	0.056	2.311	0.129
(*N* Mothers = 185)	Mother's diet (low)	0.000	0.110	0.000	0.998
	Mother's inbreeding status (inbred)	−0.072	0.105	0.468	0.494
Random effects	Mother's ID variance	0.055			
	Block variance	0.015			
	Residual variance	0.968			

Table 1 Maternal effects on offspring performance: results from Experiment 1. Results from final mixed models with parameter estimates and chi-squared (χ^2) tests for effects of sex of the offspring, mother's age since maturity, diet and inbreeding status; nonsignificant interactions were dropped from the final models.

P-values in bold are statistically significant. All analyses were performed on standardized response variables; sample sizes are shown for each response variable. For two-level factors, the parameter shown is the effect of the variable level shown, relative to the other.

$P > 0.322$). Inbred fathers sired sons with a relatively shorter gonopodium ($P = 0.012$; Table 2), but if values more than 2 SD from the mean are excluded (9 of 181 sons, from 9 fathers), the effect was not significant (GLMM: $\chi^2_{(1)} = 2.717$; $P = 0.099$; $n = 172$).

Discussion

We explored the action of three factors that generate parental effects in *Gambusia holbrooki*. A mother's age, a father's early diet and a father's inbreeding status affected offspring traits such as size at birth, size and age at maturity, and sons' genital length. Parental diet and inbreeding status were experimentally manipulated, so we can assign a direct causal role to each factor. However, the way in which these parental effects occurred was complex. First, the factors, or combinations of

factors, causing parental effects differed between mothers and fathers. Second, some parental effects differed for daughters and sons. Third, different factors, or combinations thereof, influenced how parental effects manifested for each offspring trait that we examined.

Comparing maternal and paternal effects: the case of age

Older mothers and older fathers both had offspring that were smaller at birth. Although the paternal effect was marginally nonsignificant ($P = 0.054$), the estimates for the effect of paternal age were remarkably similar to the estimates for maternal age (see Tables 1 and 2), suggesting that the difference in significance was due to a smaller sample size for fathers (the power to detect an effect equivalent to that for mothers was 52%).
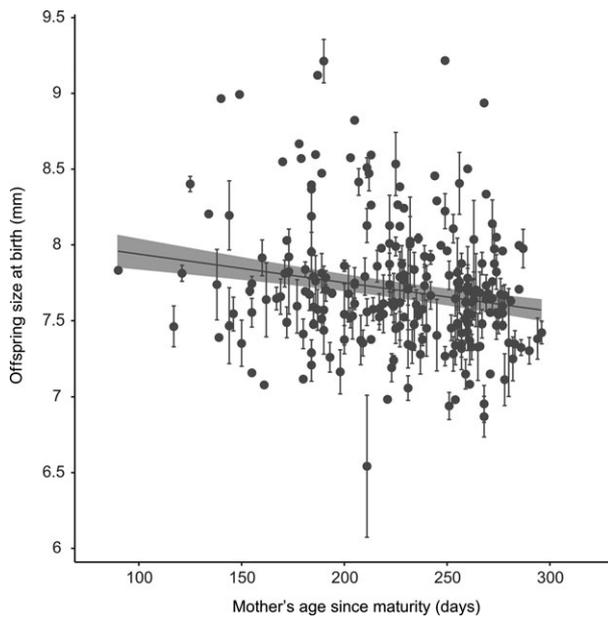
Fig. 2 Offspring size at birth. The effect of maternal age since maturity (in days) on the size of offspring at birth. Each data point represents the mean for each family (mothers $N = 226$) with SE. The line represents model predictions, with grey shading showing 95% confidence intervals around the regression.

Reports of negative effects of parental age on offspring phenotype are common (e.g. maternal age effects: Hercus & Hoffmann, 2000; Benton *et al.*, 2008; paternal age effects: Ducatez *et al.*, 2012; Nystrand & Dowling, 2014). Our results are surprising, however, because maternal effects are expected to be stronger than paternal effects when there is no male parental care (Curley *et al.*, 2011; Crean *et al.*, 2013). This is because mothers have greater contact with developing offspring (e.g. gestation) and only eggs contribute substantial material resources to zygotes. Although there is no evidence in *G. holbrooki* that mothers transfer nutrients to offspring after egg fertilization (Pollux *et al.*, 2014), older mothers might provide fewer resources to eggs, thereby affecting offspring birth size. But what about older males? Previous studies on poeciliids, including mosquitofish, show that sperm quality declines both with paternal age (Vega-Trejo *et al.*, 2016b) and with sperm age (i.e. sperm storage; Gasparini *et al.*, 2010). Additionally, there is evidence in poecillids that male offspring arising from parents with aged sperm suffer from impaired sperm quality (Gasparini *et al.*, 2017). It is unknown whether this decline is due to changes in ejaculate composition or in the sperm themselves (e.g. epigenetic factors such as DNA methylation, or mutations), but our results suggest that these changes might be as influential
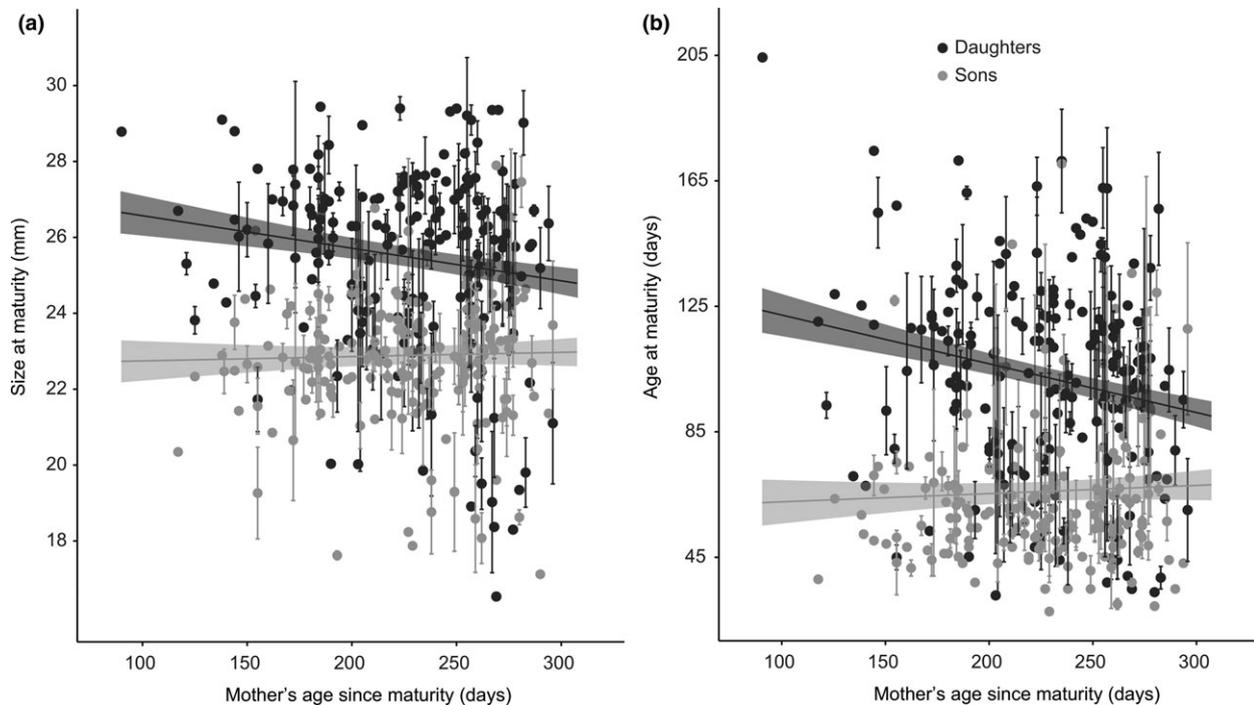

Fig. 3 Offspring size and age at maturity. The effect of maternal age on sons' and daughters' a) size and b) age at maturity. Each data point represents the mean for each family (mothers $N = 226$) with SE. Black symbols and lines represent daughters, grey symbols and lines represent sons. Lines are based on model predictions. Grey shading represents 95% confidence intervals.

Response variable	Predictor	Estimate	SE	χ^2	P
Size at birth	Intercept	0.290	0.170	2.904	0.088
(N offspring = 344)	Sex (male)	−0.138	0.082	2.878	0.090
(N Fathers = 83)	Father's age since maturity	**−0.179**	**0.093**	**3.704**	**0.054**
	Father's diet (low)	−0.083	0.184	0.205	0.651
	Father's inbreeding status (inbred)	−0.072	0.156	0.215	0.643
Random effects	Mother's ID variance	0.383			
	Father's ID variance	0.000			
	Block variance	0.115			
	Residual variance	0.664			
Size at maturity	Intercept	−0.429	0.130	10.906	0.001
(N offspring = 343)	Sex (male)	−0.048	0.087	0.309	0.578
(N Fathers = 84)	Father's age since maturity	−0.033	0.071	0.217	0.642
	Father's diet (low)	0.166	0.141	1.374	0.241
	Father's inbreeding status (inbred)	−0.011	0.118	0.009	0.925
Random effects	Mother's ID variance	0.108			
	Father's ID variance	0.017			
	Block variance	0.041			
	Residual variance	0.741			
Age at maturity	Intercept	−0.349	0.148	5.542	0.019
(N offspring = 346)	Sex (male)	0.145	0.092	2.469	0.116
(N Fathers = 84)	Father's age since maturity	−0.080	0.074	1.193	0.275
	Father's diet (low)	**0.352**	**0.148**	**5.674**	**0.017**
	Father's inbreeding status (inbred)	0.119	0.120	0.980	0.322
Random effects	Mother's ID variance	0.054			
	Father's ID variance	0.024			
	Block variance	0.143			
	Residual variance	0.800			
Relative gonopodium size	Intercept	0.071	0.174	0.168	0.682
(N offspring = 181)	Father's age since maturity	**0.344**	**0.167**	**4.270**	**0.039**
(N Fathers = 69)	Father's diet (low)	0.014	0.195	0.005	0.942
	Father's inbreeding status (inbred)	**−0.424**	**0.169**	**6.318**	**0.012**
	Father's diet × Father's age since maturity	**−0.627**	**0.213**	**8.654**	**0.003**
Random effects	Mother's ID variance	0.169			
	Father's ID variance	0.000			
	Block variance	0.000			
	Residual variance	0.885			

Table 2 Paternal effects on offspring performance: results from Experiment 2. Results from final mixed models with parameter estimates and chi-squared (χ^2) tests for effects of sex of the offspring, father's age since maturity, diet and inbreeding status; nonsignificant interactions were dropped from final models.

P-values in bold are statistically significant. All analyses were performed on standardized response variables. Sample sizes are shown for each response variable. For two-level factors, the parameter shown is the effect of the variable level shown relative to the other.

in determining offspring size at birth as those arising from maternal effects (see also Preston *et al.*, 2015). A direct comparison between maternal and paternal effects in our study has to be made with caution though because of: the slightly different structure of the random effects in our models; potential differences in the rearing conditions of the stock fish who were parents in each of our experiments; and differences in how offspring were 'created' (i.e. mating for maternal effects, and artificial inseminations for paternal effects). Nevertheless, our study adds to recent evidence that paternal effects might be as important as maternal effects in some species (Curley *et al.*, 2011; Crean & Bonduriansky, 2014; Evans *et al.*, 2017).

Parental effects: sons vs. daughters

The effects of maternal age on offspring size and age at maturity were sex-specific. Older mothers had smaller daughters that matured more quickly, but there was no such effect for sons. Previous studies of the same mosquitofish population (Kruuk *et al.*, 2015; Vega-Trejo *et al.*, 2018) found considerable variation among mothers in maternal effects for the size and time to maturity of both sons and daughters. Intriguingly, however, there was no correlation between the effect a mother had on her sons vs. her daughters (e.g. mothers that produced larger daughters did not produce larger sons; Kruuk *et al.*, 2015). Sex-specific maternal effects have
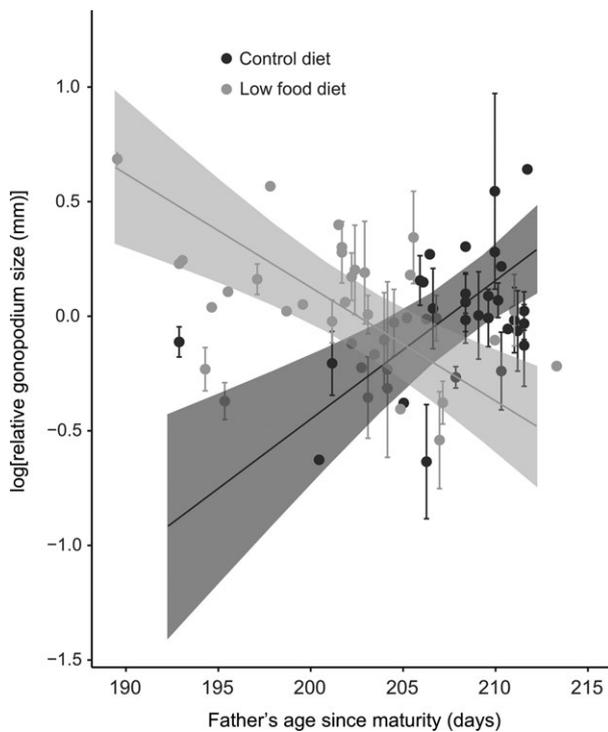
Fig. 4 Offspring relative gonopodium size. The effect of paternal age and diet on their offsprings' relative gonopodium size. Each data point represents the mean for each family (fathers $N = 69$) with SE. Black symbols and lines represent sons from fathers on the control diet, grey symbols and lines represent sons from fathers on the low-food diet. Lines are based on model predictions. Grey shading represents 95% confidence intervals.

also been reported in other species. For example, in seed beetles there were maternal effects on the lifespan of sons, but not of daughters (Fox *et al.*, 2004), and in red deer, there were maternal effects for longevity and breeding success for daughters but not for sons (Kruuk *et al.*, 2000). It is known that optimal developmental conditions differ for males and females due to divergent selection (Uller, 2008). Similarly, parental effects can generate sex-specific effects in their offspring.

Parental effects as offspring grow

Although mothers and fathers had similar effects on off-spring size at birth, as offspring grew these effects diverged for sons and daughters. In general, the importance of parental effects declines in older offspring (Lindholm *et al.*, 2006; Wilson & Reale, 2006). This may be because variation in resources available later in life masks parental effects (Monaghan, 2008; Auer, 2010) or because compensatory growth can act to eliminate initial differences in body size (Metcalfe & Monaghan, 2001; Hector & Nakagawa, 2012). It is also possible that parental effects are actually stronger earlier when, for instance, maternal investment in egg content directly affects offspring

(Bernardo, 1996a). However, we found that, in addition to parental effects being less prevalent in older offspring (Tables 1 and 2), they tended to become more complex. For example, the negative effect of maternal age on off-spring body size and time to maturation only persisted for daughters; and for fathers, the effect of paternal age on relative gonopodium length was moderated by the father's rearing diet. Our results highlight the wider need to account for transgenerational effects when measuring fitness traits (see also Bouwhuis *et al.*, 2015), and to consider how multiple factors interact to generate parental effects.

Parental effects and inbreeding

The negative effects of being inbred on an individual's performance are well established (review: Hedrick & Kalinowski, 2000; Keller & Waller, 2002), so it is tempting to assume that parental inbreeding status must have consequences for parental effects that thereby elevate the net cost of inbreeding (Huisman *et al.*, 2016). In an earlier experiment, we found that being inbred significantly lowered male reproductive success in *G. holbrooki* (Vega-Trejo *et al.*, 2017) suggesting that it lowers fitness. Even so, there was no detectable inbreeding depression for a range of major life history (Vega-Trejo *et al.*, 2015, 2016a) and reproductive traits in this system (sperm number, sperm velocity and gonopodium length; Vega-Trejo *et al.*, 2016b; Marsh *et al.*, 2017). Similarly, there was almost no effect of parental inbreeding status on off-spring performance in the current study. The only exception was that inbred fathers sired sons with a relatively shorter gonopodium, even though they themselves did not have a shorter than average gonopodium (Vega-Trejo *et al.*, 2017). This parental effect could potentially lower a son's fitness as relative gonopodium length predicts reproductive success in *G. holbrooki* (Head *et al.*, 2017; Vega-Trejo *et al.*, 2017; but see Booksmythe *et al.* 2016). However, we treat our finding about sons' gonopodium length with caution given the weak statistical support for this claim (Table 2), and the dependence of the statistical significance on nine (of 181) individuals. Therefore, we are unsure what drives this effect and believe this result is worth replicating before placing too much weight on it. It is worth noting that previous studies showing that parental inbreeding affects offspring phenotypes have mainly been on species with post-natal parental care (e.g. Mattey *et al.*, 2013; Bérénos *et al.*, 2016; Huisman *et al.*, 2016; Pilakouta & Smiseth, 2016), although some studies have shown effects of parental inbreeding on offspring performance in insects lacking parental care such as *Drosophila* (see Tan *et al.*, 2013; Nguyen & Moehring, 2017).

Conclusion

Quantitative genetic studies frequently consider the proportion of phenotypic variance that is due to

maternal effects (Falconer & Mackay, 1996; Kruuk & Hadfield, 2007), but both maternal *and* paternal effects can contribute to characteristics of offspring phenotype. The effect of factors that affect parental phenotypes should be investigated for both mothers and fathers (Mousseau & Fox, 1998; Santure & Spencer, 2006). In addition, even when maternal and paternal effects are documented, the underlying causes of variation in these parental effects typically remain unknown (Crean & Bonduriansky, 2014; van den Heuvel *et al.*, 2016). Here, we took an experimental approach to test the extent to which parental inbreeding status, parental diet and parental age generate parental effects in *G. holbrooki*. The observed parental effects depended on both parental and offspring sex, and on interactions between offspring sex and parental age, diet and inbreeding status. Separating the influence of these factors was facilitated by our simple experimental set-up (a two-by-two factorial design) that removed confounding correlations with unmeasured variables. Our study adds to the growing evidence for complex parental effects, even in species lacking parental care. Given the nature of the patterns we found, our findings raise new questions about the underlying proximate mechanisms responsible for the sex-specificity of parental effects.

Acknowledgments

Funding

Data accessibility

All data associated with this study have been deposited in the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.6642j2s.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Data S1 Supplementary material.

Table S1 Means ± SD (*N* of offspring/*N* of parents) from raw data separated by parent's diet treatment for age since maturity and chronological age (both in days).

Table S2 Means ± SE (*N* of offspring/*N* of parents) from raw data separated by offspring sex, parent's inbreeding status, and parent's diet for a) maternal effects experiment and b) paternal effects experiment.

Table S3 Maternal effects on offspring performance in models using chronological rather than age since maturity (c.f. Table 1).

Table S4 Paternal effects on offspring performance in models using chronological rather than age since maturity (c.f. Table 2).

Table S5 Maternal effects on offspring performance in models including mother's size when giving birth.

Table S6 Paternal effects on offspring performance in models including father's size when inseminating females.