An experimental test of the role of male mating history on paternal effects in the livebearer fish, *Gambusia holbrooki*

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Abstract

Studies often show that paternal age affects offspring fitness. However, such effects could be due either to age, or to a male's previous mating effort (which is necessarily confounded with age). We experimentally tested whether differences in the mating history of old males affects offspring performance in the mosquitofish, *Gambusia holbrooki*. Upon maturation, males were housed for a duration of natural field-breeding season (23-weeks) either with mating access to females ('lifetime-mating'), or with visual but no physical access to females ('no-mating'). We then paired these males with a female to test whether male mating history had significant effect on their mate's breeding success or offspring performance. The daughters, but not the sons, of 'no-mating' treatment males matured significantly sooner, and at a significantly smaller size, than those of 'lifetime-mating' treatment males. There was, however, no effect of male mating history on their daughters' initial fecundity, or on proxy measures of their sons' reproductive success. These results, when combined with earlier studies showing effects of male mating history on sperm quality, growth and immunity, suggest that variation in paternal effects currently attributed to male age could partly arise because older males have usually mated more often than younger males.

Keywords

ageing, mating history, paternal effects, poeciliids, sperm quality, sexual selection

Introduction

Numerous studies on human and other animals have investigated whether a father's age is associated with offspring performance [1-4]. Special attention has been paid to cases where a relationship cannot be attributable to a reduction in male parental care. In most cases there is a reported decline in offspring performance with paternal age [1-3]. For example, offspring sired by older males more often have health disorders in humans [5,6], reduced early embryo survival in cabbage beetles [7], slower growth and reduced longevity in mice [8,9], lower fecundity in bulb mites [10], and higher mortality in ungulates [11]. These declines are attributed to offspring inheriting mutations accumulated in germline of older males [5,12,13], to epigenetic changes, or to substances transferred in ejaculates that alter gene expression in offspring [9,14,15]. Fewer studies have, however, also report that male age has positive effects on offspring (e.g. mating with older males increases egg hatching success in insects [16,17], and juvenile survival in fruitflies [18,19]). But to what extent does male age, rather than a factor that tends to covary with age, explain the general trend for a negative correlation between male age and offspring fitness?

A key factor that might determine how paternal age affects offspring success is male's past mating activity [2]. In general, older males are likely to have mated more often than younger males [20,21]. The resources invested to acquire mating, produce sperm and so on, impose energetic and maintenance costs (i.e. reproductive effort costs) that might lower a male's ability to repair germline DNA [15,22,23, but see 24]. This could lead to age-dependent paternal effects. To determine whether male mating histories actually have causal effects on offspring performance it is necessary to conduct experiments. We need to manipulate male mating history and then test for an effect on offspring performance. To date, few such experiments have been conducted. In most studies age and mating history are conflated (e.g.

observational studies of birds). Here we therefore focus on testing for a direct effect of male mating history while controlling for male age.

In the eastern mosquitofish (*Gambusia holbrooki*), we control for any effect of paternal age by only using old males as sires. We calculated the effect of an experimental manipulation of these males' mating history on their subsequent fertility and on components of offspring fitness. Recently matured males were housed for 23 weeks either with access to females with whom they could mate ('lifetime mating treatment'), or with only visual access to females ('no mating treatment'). We then paired old males with a female to test for any effect of male mating history on their mate's fecundity (brood size) and their offsprings' reproductive performance (sons' mating potential and daughters' initial fecundity).

Materials and Methods

Origin and maintenance of animals

Juvenile male *Gambusia holbrooki* (n=144) were collected from the wild. Upon reaching sexual maturity (at approx. 6-8 weeks of age), males were randomly allocated to one of two mating treatments for a period of 23-25 weeks. Half the males were individually housed in 7*l* aquaria with a female with whom they could mate freely ('lifetime mating treatment'). The other half were individually housed in 7*l* aquaria with a female behind a mesh barrier: they had access to visual and olfactory cues from females, but could not mate ('no mating treatment') (figure1, also see [21]). For both treatments, females were rotated between tanks weekly to maintain male sexual interest.

Study design

When males were 24 weeks old they were removed from their individual treatment tank. We then created trios of three males of the same treatment type: 23 lifetime mating and 25 no mating treatment trios. Each trio of males was then introduced into a 7*l* aquarium, along with a virgin female. These females were the lab-born offspring of wild caught mothers, reared in laboratory and held in single-sex groups (40 fish/90l aquaria) from maturity to ensure virginity. We used three rather than one male per female to ensure natural levels of polyandry [26].

Female reproductive output and offspring growth

After 20 days, the 48 females were transferred to individual 1*l* tanks (gestation is >21 days) containing plastic mesh refuge to protect offspring from matricide. They were checked twice daily and we recorded the date of birth and number of offspring. In total, 19 of 23 females housed with lifetime mating treatment males and 22 of 25 housed with no mating treatment males bred. Up to 10 fry/brood were photographed to measure their standard length at birth (n=251). Offspring from 30 broods (n=14 lifetime mating; 16 no mating treatment) were then reared individually and re-photographed at 21 days of age (n=199) to calculate their early growth rate [26]. Not all broods were retained due to logistic constraints.

Offspring reproductive performance

To test if paternal mating history, controlling for paternal age, affects offspring reproductive performance, we reared sons and daughters to maturity in their individual 1*l* tanks (see Supplementary Materials for details). Each individual was photographed at maturity to measure its standard length and, for males, also their relative gonopodium length (a predictor of male insemination success [27]). At a standardised age of five weeks post-maturity, we measured traits likely to be linked to reproductive success. In case of daughters (n=103), we counted the number of eggs available for fertilisation (i.e. their initial fecundity). We also photographed eggs under a dissecting microscope alongside a reference scale, and measured the diameter of five randomly chosen eggs using *ImageJ*. The mating potential of sons was estimated in two behavioural assays made five weeks after maturation (n=81). First, we measured attractiveness in two-choice association trials where test females chose between the focal male and a stock male [28]. Second, we measured male mating behaviour (e.g. time near female, number of copulation attempts) when the male freely interacted with the test female for 10 mins (see Supplementary Materials). After the mating behaviour measures were taken, sons were returned to their individual tanks for 7d to allow for sperm replenishment [28]. Finally, we recorded sperm number and sperm swimming velocity as proxies for the sons' potential to achieve fertilisation success under sperm competition (female *G. holbrooki* mate multiply) [21,29]. We make standard assumption based on results in many species that males with more sperm and faster swimming sperm are more likely to gain paternity when there is sperm competition.

All data were collected blind to male mating treatment. All fish were eventually euthanized in MS222 to comply with Australian legislation prohibiting the release of pest species.

Statistical analyses

The effect of a male's mating treatment on female reproductive success was evaluated using three response variables: 1) whether or not a female gave birth (yes/no); 2) gestation period;

and 3) brood size. The effect of male mating treatment on offspring was evaluated using: 4) size at birth; 5) early growth rate; 6) early survival, and 7) size at maturity; and for daughters: 8) adult growth; 9) fecundity; and 10) egg size; and for sons: 11) 'mating behaviours'; 12) relative gonopodium length (residuals of log-log regression on standard length); 13) sperm velocity; and 14) sperm count. 'Mating behaviours' was the first principle component extracted from information on male attractiveness in two choice trials and three mating behaviours (see Supplementary Material). We also tested for an effect of male mating treatment on the offspring sex ratio.

We ran generalized linear, generalized linear-mixed and linear-mixed effect models in R v3.6.0 [30]. In all models, male mating treatment ('lifetime mating' or 'no mating') was a fixed effect, and, where relevant, female body size was a covariate. When analysing post-maturation offspring traits, we included offspring size as a covariate and the interaction between male mating treatment and offspring sex. In all models for offspring traits we included maternal ID as a random factor because we measured several offspring per brood. The supplementary material contains further details about the methods and analyses.

Results

Summary statistics and model parameter estimates for the effect of male mating treatment on female fecundity and offspring performance are shown in table 1. There was no effect of male mating treatment on the probability that a female gave birth, her gestation period or brood size; nor was there any effect on offspring size at birth, early survival, or early growth rate. Male mating treatment also had no effect on offspring sex ratio ($\chi 2= 0.133$, df= 1, p= 0.715).

There was a clear sex-specific effect of male mating treatment on both time to, and size at, maturation (mating treatment*sex, both $p<0.01$). The daughters of no mating treatment males matured significantly sooner, and at a smaller size, than those of lifetime mating treatment males. There were no such effects on the size and time to maturation of sons (figure 2a,b).

There was no effect of male mating treatment on daughters' growth, number of eggs or egg diameter; nor were there any effects on sons' sperm count, sperm velocity, relative gonopodium length, or mating behaviour (table 1). Details are provided in tables S1, S2.

Table 1. Parameter estimates and test statistics for the effect of male mating treatment on female reproductive output and offspring traits in eastern mosquitofish (*G. holbrooki*). Mating treatment values are for ‘no mating’ treatment. Offspring sex values are for sons. Full model outputs are provided in the supplementary material, Tables S1, S2.

Trait	**Predictor**	**Estimate**	**SE**	**Test statistic**		***P***
Female fecundity						
Bred (Yes/No)	Mating treatment	-0.111	0.832	χ2	0.018	0.894
Gestation period	Mating treatment	-0.010	0.059	χ2	0.029	0.864
Brood size	Mating treatment	0.156	0.192	χ2	0.656	0.418
Offspring traits						
Size at birth	Mating treatment	-0.107	0.121	F	0.784	0.382
	Sex	0.118	0.088	F	1.781	0.184
	Mating treatment *Sex	-0.134	0.114	F	1.361	0.245
Survival to 21d	Mating treatment	0.179	1.167	χ2	0.023	0.878
Early growth	Mating treatment	0.001	0.024	F	0.002	0.968
	Sex	-0.026	0.013	F	4.231	**0.041**
	Mating treatment *Sex	0.029	0.016	F	3.073	0.082
Size at maturity	Mating treatment	-1.238	0.627	F	3.881	0.057
	Sex	-1.744	0.470	F	13.57	**<0.001**
	Mating treatment *Sex	1.720	0.607	F	7.921	**0.005**
Time to maturity	Mating treatment	-0.130	0.048	χ2	7.466	**0.006**
	Sex	-0.020	0.027	χ2	0.577	0.447
	Mating treatment *Sex	0.118	0.035	χ2	11.41	**<0.001**
Daughter traits						
Egg Number	Mating treatment	0.950	1.155	F	0.823	0.422
Egg size	Mating treatment	-0.023	0.049	F	0.212	0.650
Adult growth rate	Mating treatment	0.005	0.010	F	0.238	0.630
Son traits						
Sperm velocity	Mating treatment	-3.161	3.797	F	0.656	0.431
Sperm count	Mating treatment	25.97	146.97	F	0.030	0.866
Gonopodium size	Mating treatment	-0.004	0.008	F	0.288	0.599
Mating behaviour	Mating treatment	-0.523	0.291	F	3.061	0.099

Discussion

Many studies have focused on the effect of male age on reproductive traits, such as sperm count and mating success [2]. Fewer studies look at the effects on offspring fitness [4,30], but almost none of these studies have conclusively shown that male age itself affects offspring performance. This is because age is always confounded with other variables, most notably a male's mating history. We therefore experimentally tested for the effect of lifetime mating activity on the offspring performance of old males of same age [20,21].

We manipulated the access of male *G. holbrooki* to females over their natural adult lifespan to test whether, for old fathers, total lifetime mating activity affects their offspring. Any effect of mating activity is presumably due to either the transmission of non-genetic information from father to offspring, or because greater mating activity increases the rate of inheritance of germline mutations [14,15,31]. We hypothesised that males who had been prevented from mating prior to breeding would produce higher performing offspring than males who had experienced a lifetime of mating activity. In partial support of this prediction we found a strong effect of fathers' mating history on their daughters' maturation rate. The daughters of males with no previous mating activity matured significantly sooner, albeit at a smaller size (1mm smaller which is unlikely to have a large effect on fecundity), than the daughters of males who had experiencing a lifetime of mating (both $P<0.01$). This suggests that a father's mating history might alter traits potentially linked to the fitness of his daughters. In contrast, we did not find any effect of paternal mating activity on the putative fitness-related traits that we measured in sons. There was no difference in sperm traits, morphology or mating behaviour between the sons of males with a lifetime of mating activity or no prior mating activity. Our results, in conjunction with other studies, suggest that cross-generational paternal effects on traits often linked to fitness (such as body size) can be sex-specific [32,33]. The mechanisms that generate sex-specific paternal effects are largely unknown, but they include differences in the timing and plasticity of events during gamete maturation, and epigenetic changes in gene expression on sex chromosomes unique to males and females [33,34].

Many studies have shown that a male's mating history can affect the fitness of females with whom he mates [34,35]. We did not, however, observe any effect of a male's past mating activity on female breeding success in *G. holbrooki*. One explanation could be that studies investigating the effect of male mating history on female reproductive output mainly use insects where ejaculates provide nutrients to females [35,36]. In contrast, in *G. holbrooki*, females do not receive any obvious direct nutritional benefits from males. Our finding is consistent with our recent study where female *G. holbrooki* housed with either a large or small male (where larger males have bigger ejaculates [37]) showed no difference in reproductive output [38]. Finally, there might a publication bias against non-significant results obscuring evidence that male mating history does not affect female reproductive output [39].

The current study, when combined with our previous work showing that male mating activity affects sperm traits and proxies of male condition (e.g. immunity) in *G. holbrooki* [21,25], highlights the wider difficulty of directly attributing poor performance by the offspring of older males to the age of their father. Male age and mating activity are naturally confounded. Here we have not directly investigated the effect of male age. Ideally, future studies should examine the independent main effects of male age and mating activity in males that are young or old (i.e. in a 2x2 design). Only then can we determine the relative role of past mating activity and male age on the fitness of a male's progeny.

Figure legends

Figure 1. Experimental protocol to determine how male mating history affects offspring performance in eastern mosquitofish (*Gambusia holbrooki*). Females are represented by fish with a black gravid spot, and males by fish with an extended anal fin (the gonopodium).

Figure 2. *Gambusia holbrooki*, the effect of mating history of old fathers on offspring reproductive traits: (a) the time (in days) for daughters (n=103, Venus symbol) and sons (n=81, Mars symbol) of fathers experiencing either 'lifetime mating' or 'no mating' to reach sexual maturity; and (b) the body size (standard length in mm) of these daughters (n=103, Venus symbol) and sons (n=81, Mars symbol) at sexual maturity. Box-plots show median (black line) and interquartile range of data.

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Figure 1. Experimental protocol to determine how male mating history affects offspring performance in eastern mosquitofish (*Gambusia holbrooki*). Females are represented by fish with a black gravid spot, and males by fish with an extended anal fin (the gonopodium).

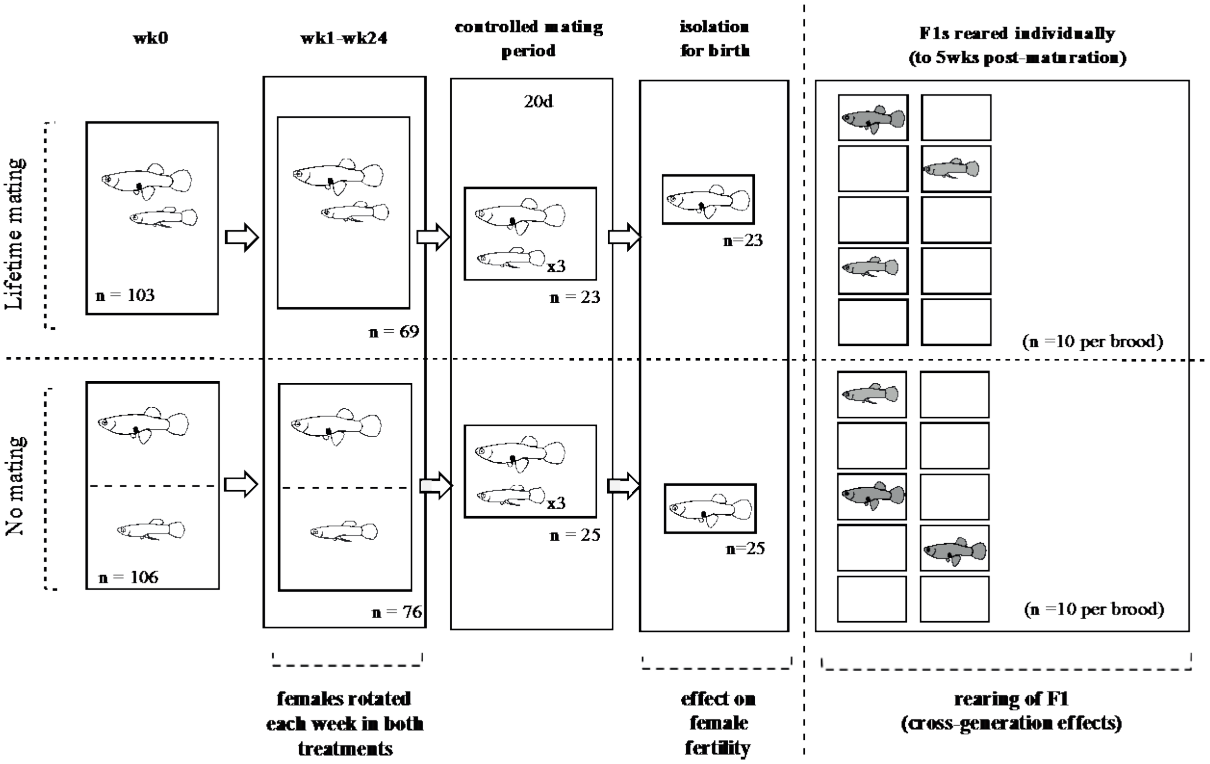

Figure 2. *Gambusia holbrooki*, the effect of mating history of old fathers on offspring reproductive traits: (a) the time (in days) for daughters (n=103, Venus symbol) and sons (n=81, Mars symbol) of fathers experiencing either 'lifetime mating' or 'no mating' to reach sexual maturity; and (b) the body size (standard length in mm) of these daughters (n=103, Venus symbol) and sons (n=81, Mars symbol) at sexual maturity. Box-plots show median (black line) and interquartile range of data.