Condition and reproductive investment in the western mosquitofish (*Gambusia affinis*): little evidence for condition-dependent sex-biased investment

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In sexually reproducing species, resources may theoretically be distributed with bias to the production of male or female offspring in response to the condition of the mother, commonly recognized as sex allocation. Using a recently characterized sex-specific molecular marker, we tested for maternal sex allocation (i.e. maternal primary sex ratio bias and sex-specific offspring investment) in captive laboratory-bred western mosquitofish (*Gambusia affinis*) at early stages of offspring development. We found no statistical evidence to support sex allocation in *G. affinis*, based on maternal condition. In addition, we found little evidence for correlations between maternal condition and investment in the condition (mass) of individual offspring (of one sex or the other), although we did find that larger mothers tended to have higher fecundity. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **119**, 430–435.

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INTRODUCTION

In many species, it is predicted that it may be adaptive for a mother to bias the allocation of her resources to the production of offspring of one sex over the other in response to her own condition (Charnov, 1982; West, 2009). This type of condition-dependent sex allocation may manifest as a manipulation of offspring sex ratio (e.g. overproduction of one sex) and/or a form of sex-biased investment (e.g. producing one sex at a 'higher quality'; Charnov, 1982; West, 2009). *Sensu* Fisher (1930), Hamilton (1967) laid the foundation for a number of subsequent hypotheses on sex allocation (West, 2009).

Among the most commonly studied hypotheses of sex allocation is the Trivers–Willard hypothesis (TWH) (West, 2009). In its most simplistic form, the TWH posits that, assuming maternal condition correlates strongly with offspring condition, mothers in good condition should produce offspring of the sex with the greatest variance in fitness (Cameron, 2004). For example, in a polygynous system where a male can monopolize mating, sons in good condition may be of higher reproductive value than daughters. Accordingly, in this system, mothers in good condition should bias investment in sons (Trivers & Willard, 1973). In the same system, mothers in poor condition should produce daughters because daughters in poor condition would be of some reproductive value, whereas sons in poor condition are unlikely to reproduce. Alternatively, in species where females

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are the larger sex and fecundity correlates with size (Shine, 1988; Honěk, 1993), if we assume that mothers in better condition produce larger daughters, we might expect daughter-biasing by mothers in good condition. In addition to these linear relationships between maternal condition and sex-biased offspring investment, we might expect more complex nonlinear relationships depending on relative reproductive values of sons and daughters based on their condition (Schindler *et al.*, 2015).

Poeciliidae is a family of live-bearing freshwater fish, which is of particular interest to the topic of sexual-size dimorphism, mating systems, and sex allocation (Bisazza, 1993). In the present study, we focus on the western mosquitofish (*Gambusia affinis*), a species with a female heterogametic sex-determination (ZW) system, for which a molecular sex marker, Gaff88, was recently developed (Lamatsch *et al.*, 2015). In *G. affinis*, reproductive success appears to be skewed in favour of larger socially dominant and aggressive males (Deaton, 2008a), perhaps giving rise to son-biasing. That being said, smaller males may use a 'sneaker' strategy and multiple paternity is relatively common, meaning that *G. affinis* may be considered promiscuous rather than polygynous (Hughes, 1985; Deaton, 2008b) undermining the theoretical basis for son-biasing based on conventional TWH-type arguments. Alternatively, it may be argued that daughter-biasing by female *Gambusia* in good condition is to be expected. Positive associations between female size and fecundity are well known in *Gambusia* and other Poecillids (Reznick, Callahan & Llauredo, 1996; Marsh-Matthews *et al.*, 2005; Kindsvater, Rosenthal & Alonzo, 2012). Importantly, recent work in *Gambusia holbrooki* (the sister species of *G. affinis*, with a male heterogametic, or XY, sex-determination system) detected a tendency towards daughter-biasing by larger mothers (Kahn, Kokko & Jennions, 2013).

The main aim of the present study was to test for deviations from an equal offspring sex ratio and for sex-specific investment (either son or daughter-biasing) as a function of maternal condition in *G. affinis*, as well as associations between maternal and offspring condition. We used the recently developed marker Gaf88 to assess primary sex ratios at early stages of development, thus avoiding later stage biases such as *ex utero* sex-biased mortality. We also report the overall association between maternal size and fecundity.

MATERIAL AND METHODS

ETHICAL STATEMENT

All experimental procedures in this research were conducted in accordance with the principles outlined in the University of Otago's Code of Ethical Conduct for the Manipulation of Animals. Approvals for all experimental protocols were granted by the University's Animal Ethics Committee (AEC, Permit Number 40/11). Dissections were performed after individuals had been euthanized (via cervical dislocation) and all efforts were made to minimize suffering.

STUDY SYSTEM

A founding population of around 120 juvenile *G. affinis* was caught from campus lakes at the University of Waikato (New Zealand) (37°47′13″S, 175°18′50″E) in mid-2010, and translocated to the animal facility at the Department of Zoology, University of Otago (New Zealand) (45°51′56″S, 170°30′50″E). First-generation fishes (i.e. the offspring of founders) were allowed to interact freely in a single 180-litre tank (density of approximately 0.75 fish/litre, with an approximately equal sex ratio) and kept under a 12 : 12 h light/dark photocycle, at a water temperature of 22.5 ± 2°C. Water and filter changes were conducted on a fortnightly basis. Fishes were fed twice daily (09.00 h and 15.00 h) with a mixture of dry fish food and frozen brine shrimp.

DATA COLLECTION

From these first generation fishes, we dissected females as they appeared to enter the late stage of gestation, which was identified by the presence of a heavily enlarged abdomen and gravid spot (McPeek, 1992). Any variation in female size was the result of standing variation (i.e. we did not manipulate female condition) and, given that it was not possible to mark all individuals, we could not control individual age. Each animal was euthanized and wet weight and total length measured (i.e. the length from the tip of the snout to the tip of the longer lobe of the caudal fin). We counted and weighed propagules. Each propagule was then preserved in 100% ethanol for DNA extraction. We were able to dissect 28 females (i.e. propagules were successfully removed for genotyping). In addition, fecundity data (i.e. egg number but no corresponding offspring sex/weight data) were obtained from a separate dissection of 29 females from the same laboratory population.

Each propagule was subject to genetic sex determination. We used a Chelex-based DNA extraction protocol modified from that established by Walsh, Metzger & Higuchi (1991). Following instructions for MyTaq (Bioline), 1.0 µL of solution containing DNA (approximately 100 ng/µL) was placed in each well of a polymerase chain reaction (PCR) plate. Extracted DNA samples were then left to dry overnight at room temperature (i.e. PCR DNA dry plate). The next day,

a PCR reagent mix of the following composition was prepared: 25% MyTaq mix, 5% Gaf88_F (forward primer) and 5% Gaf88_R (reverse primer) (Lamatsch *et al.*, 2015) of 10 μM concentrations and 65% MilliQ water (Millipore). We added 10 μL of this PCR mix to each of the wells of the PCR plate, which were sealed with mineral oil. Samples were amplified by PCR and female- and male-specific bands identified with post-PCR gel electrophoresis based on the procedures detailed in Lamatsch *et al.* (2015). Briefly, samples were amplified with the following cycle: (1) denaturation at 95°C for one 15-min cycle; (2) denaturation at 94°C for 40 × 30-s cycles; (3) annealing at 55°C for 40 × 90-s cycles; (4) extension at 72°C for 40 × 45-s cycles; and (5) final extension at 60°C for one 10-min cycle. Upon electrophoresis, female samples produce a strong band at approximately 500 bp, whereas males produce a multi-band pattern.

STATISTICAL ANALYSIS

We conducted statistical analysis using the 'glm' function in the base package, *stats*, and the function 'lmer' from the *lme4* package (Bates *et al.*, 2015) in R, version .3.2.1 (R Development Core Team, 2015). Although size is generally considered to correlate with condition, a single measure of size alone (e.g. mass or length) is not typically considered as adequate. Many studies use the residuals of mass–length regression as an index of condition; however, residual analysis can be substantially biased (Darlington & Smulders, 2001; Freckleton, 2002). Rather, in the present study, we fitted models with length and mass (*Z*-transformed) as simultaneous predictors in multivariate regression, which is less biased than residual analysis (Freckleton, 2002). Data were then analyzed using three separate models, each of which addressed a different question.

Model 1 tested whether the primary sex ratio was influenced by maternal condition by fitting a generalized linear model (GLM) with the family specified as quasi-binomial (logit-link function), where the respective count of male and female offspring within a female was the response variable, and maternal mass and length were the explanatory variables. As discussed in the Introduction, small male *Gambusia* may adopt a sneaker strategy, meaning that both small and large males are of high reproductive value. In turn, this alternative strategy may lead to what is termed a 'type 3' Trivers–Willard effect (Schindler *et al.*, 2015), where, assuming a correlation between maternal condition and offspring size, both mothers in poor condition and those in good condition are better off over-investing in sons. Thus, we also explored a version of model 1 that fitted a quadratic term for the effect of maternal mass on sex ratio.

Model 2 tested whether offspring size was a function of maternal condition and offspring sex using a linear mixed-effects model. In model 2, the response was the mass of each propagule, and the predictors were maternal length, maternal mass, and offspring sex fitted with an interaction between the latter two, and a two-level categorical predictor giving the developmental phase of the propagule (propagules at later developmental stages may be expected to weigh more; 91.6% were considered fry and 8.6% were considered eggs). Model 2 also included a random effect for maternal ID because multiple offspring came from the same mother. Finally, model 3 was used to test for the effects of maternal size on offspring number, and comprised a GLM that fitted offspring number as the response and maternal length as a predictor (note that regression between maternal mass and fecundity would be confounded by the weight of the brood) with a quasi-Poisson family (log-link function). For all model-terms, statistical significance was based 95% confidence intervals (CIs) (Nakagawa & Cuthill, 2007).

RESULTS

From 28 females, we were able to remove 463 propagules for genotyping, of which we were able to successfully identify the genetic sex for 432 (93.3%). One hundred and ninety-six propagules were male and 236 were female, indicating a slight tendency toward daughters, although an intercept only GLM suggested that this was not significantly different from equality (GLM logit intercept estimate, CI = −0.19, −0.40 to 0.03). Multiple regression estimated slight increases and decreases in the proportion of male offspring within a female's brood with increasing maternal mass and length, respectively, although these effects were not statistically significant (Fig. 1A; for full coefficients of all models, see the Supporting information, Tables S1, S2, S3, S4, S5). A model exploring the potential nonlinear effects of maternal mass on offspring primary sex ratio by fitting a quadratic term also detected no significant effects of mass or length on sex ratio.

We were able to accurately measure the mass and identify the sex of 379 propagules from 22 females (some propagules were too badly damaged during the dissection to accurately weigh; e.g. the yolk sack was burst). Among useable propagules, there was a slight positive relationship between maternal length and offspring mass, and a slight negative one between maternal mass and offspring mass, although these slopes did not significantly differ from zero, and the effects did not significantly differ between male and female propagules (Fig. 1B). Qualitatively identical
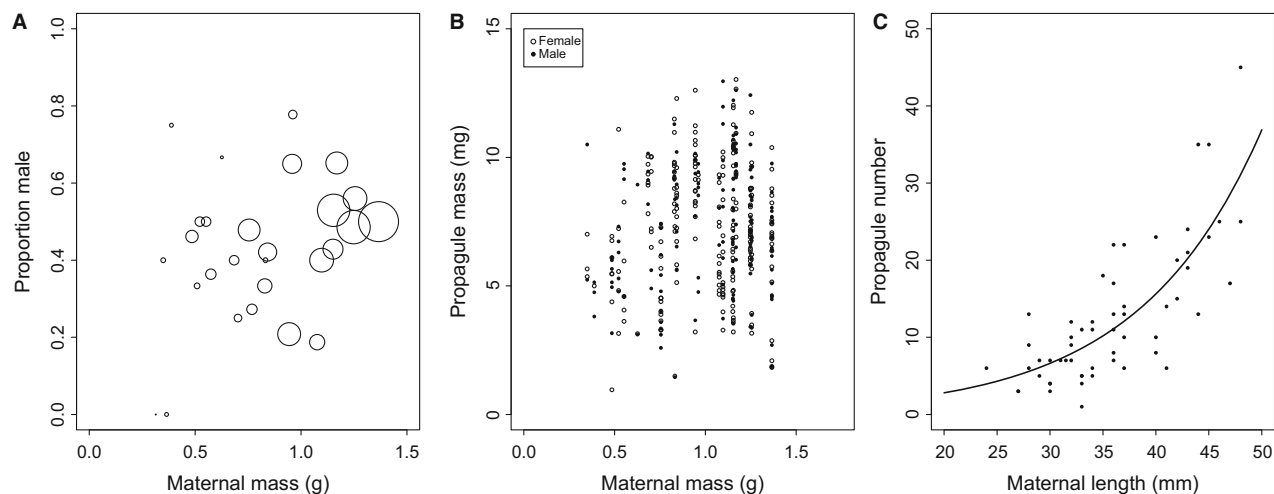
Figure 1. A, proportion of male propagules dissected out of a female, as predicted by female mass. A generalized linear model (GLM) detected a slight but nonsignificant increase in the proportion of male offspring as maternal mass increased (see Results). Circle size indicates the total number of fry obtained and sexed from a female (smallest circle $N_{offspring} = 1$, whereas the largest circle represents $N_{offspring} = 42$, $N_{mothers} = 28$, $N_{offspring} = 432$). B, offspring weight (mg) against maternal mass differentiated by sex of the offspring (open points = females, closed points = males; $N_{males} = 174$ and $N_{females} = 205$). A linear mixed-effects model estimated a slight but nonsignificant increase in offspring weight with increasing maternal mass and the difference in the magnitude of the effect between male and female offspring was not significant (see Results; $N_{offspring} = 379$, $N_{mothers} = 22$). C, number of propagules (egg and fry) as predicted by maternal length. The curve gives values as predicted GLM (see Results; $N_{mothers} = 57$).

results were also observed after controlling for maternal fecundity.

Finally, we obtained measurements of maternal length and fecundity from 57 female fish. As maternal length increased, we found that the number of propagules a fish carried increased, and the associated slope of the GLM was significantly different from zero (GLM log slope$_{Maternal\ Length}$ estimate, CI = 0.09, 0.068 to 0,104) (Fig. 1C).

DISCUSSION

We found no statistical evidence supporting condition-dependent son- or daughter-biasing in *G. affinis*, nor an association between maternal condition and offspring condition (mass). Below, we discuss three potential explanations for the lack of sex ratio-biasing in *G. affinis*. First, it is feasible that offspring sex-biasing does not occur in *G. affinis*. As discussed in the Introduction, Kahn *et al*. (2013) observe daughter-biasing in relation to maternal condition in *G. holbrooki*. Differences between our own findings and those of Kahn *et al*. (2013) may be attributable to several factors. For example, *G. affinis* may not have the ability to allocate sex, whereas *G. holbrooki* does, perhaps as a result of differences in the sex determination system (Lamatsch *et al*., 2015). Also, the present study was performed on captive laboratory-bred animals, and exploited standing variation in female size. Thus, our sampled animals may not have been subject to the necessary environmental factors (e.g. over-winter food availability) that induce sex allocation. Manipulative experimental studies in *G. affinis*, which induce variability in maternal condition and use Gaf88 to determine offspring sex ratios at early stages of development, are the natural next step. Such experiments may be able to disentangle the effects of condition, size, and age (perhaps through variation in diet), all of which may be expected to influence reproductive investment. Alternatively, applying Gaf88 to gravid wild-caught females sampled during different seasons may also be productive.

Second, *G. affinis* may bias offspring sex ratio at a level undetectable with our sample size. It has been suggested that, at the population level, humans bias the sex ratio at birth to a very small degree (Gelman, 2007; Gelman & Weakliem, 2009); a 1% sex skew at birth across latitudinal gradients has been suggested in humans (Navara, 2009). Detecting a 1% effect with 80% statistical power would require a sample size of approximately 20 000. Such a sample size is infeasible for this kind of empirical study, although a 1% skew may be important over evolutionary time. Having said that, previous studies with rather limited samples sizes have detected offspring sex ratio biases in relation to male attractiveness (Booksmythe *et al*., 2015), including in another Poeciliid,

the guppy (*Poecilia reticulata*, $N = 37$ and $N = 30$; Karino & Sato, 2009; Sato & Karino, 2010). Interestingly, similar to the guppy, female *G. affinis* are also considered to show mate preference (Deaton, 2008b). Future studies may combine trials of male attractiveness with Gaf88 to test for sex allocation in response to mate attractiveness in *G. affinis*.

Third, recent theoretical work demonstrates that measuring primary sex ratios alone may be insufficient to demonstrate adaptive sex allocation, even where Trivers–Willard type effects are expected based on the mating system (Schindler *et al.*, 2015). An implicit assumption of studies such as ours that use the primary sex ratio to test for adaptive sex allocation is that the ratio of the reproductive value of sons and daughters (the evolutionary currency of adaptive sex allocation) is equivalent to the sex ratio at birth (Schindler *et al.*, 2015). However, this assumption may be invalidated because the reproductive value of offspring is largely realized post-maturity, and the life-history trajectory between birth and maturity can be sex-specific (e,g, sons may have a higher rate of juvenile mortality). The true ratio of the reproductive value of son and daughters at birth can be estimated by coupling detailed species- and sex-specific demographic data with integral projection models (Schindler *et al.*, 2015). Yet, the required data do not currently exist for *G. affinis*.

We also examined correlations between maternal length and reproductive investment. Relationships between maternal size and offspring size/number appear to be inconsistent within the genus *Gambusia*. Our results are in accordance with a previous study on *G. affinis* and *G. geiseri*, which found that female size positively correlates with offspring number but not offspring size (Marsh-Matthews *et al.*, 2005). These species are in agreement with theory predicting that better resourced females should invest in greater numbers of offspring, rather than in 'higher quality' offspring (Smith & Fretwell, 1974). Many other species, however, including *G. holbrooki*, do not appear to follow this rule, with positive correlations between maternal size and offspring size being widely observed (Marsh-Matthews *et al.*, 2005; Lim, Senior & Nakagawa, 2014). Given this apparent intra-genus variation, *Gambusia* may comprise a good system for comparative experiments on reproductive investment in response to resource availability.

We found little evidence for primary sex ratio-biasing and sex-specific offspring investment in relation to standing variation in maternal condition in *G. affinis*. As one of only a handful of fish species for which a sex-specific marker has been identified, *G. affinis* will make a good model organism for further studies of sex ratio biases in early development. The next step is to combine our molecular sexing approach with systematic manipulations of female condition and to test for sex-biased maternal investment in other contexts.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Generalized linear model (logit-link function) estimates (Est.), associated standard errors (SE), and lower and upper 95% confidence intervals (LCI and UCI, respectively) for the effect of maternal mass and length on offspring sex ratio (positive estimates indicate more males).

Table S2. Generalized linear model (logit-link function) estimates (Est.), associated standard errors (SE), and lower and upper 95% confidence intervals (LCI and UCI, respectively) for the effect of maternal mass and length on offspring sex ratio (positive estimates indicate more males), including a quadratic effect of maternal mass on offspring sex ratio.

Table S3. Linear mixed model estimates (Est.) associated standard errors (SE), and lower and upper 95% confidence intervals (LCI and UCI, respectively) for predictors of offspring weight (mg). Random effects are standard deviation (as opposed to variance).

Table S4. Linear mixed model estimates (Est.) associated standard errors (SE), and lower and upper 95% confidence intervals (LCI and UCI, respectively) for predictors of offspring weight (mg), including maternal fecundity. Random effects are standard deviation (as opposed to variance).

Table S5. Generalized linear model (log-link function) estimates (Est.), associated standard errors (SE), and lower and upper 95% confidence intervals (LCI and UCI, respectively) for the effect of maternal length on offspring number.

SHARED DATA

Data deposited in the Pangaea® repository (Senior *et al.*, 2016).