

No evidence for adjustment of maternal investment under alternative mate availability regimes

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Using treatments that mimic high and low availability of reproductive males, it was found that female three-spined sticklebacks *Gasterosteus aculeatus*, previously shown to adjust their mate choices when male mates were rare, did not alter their reproductive investment strategies. These results suggest that plasticity in investment is perhaps limited by physiological requirements or dependent on relatively extreme mate availability regimes. The probability of becoming reproductive, number of clutches per season (per female), initial clutch size and mass and the timing of reproduction were all independent of the experience a female had with mate availability. This suggests that pre-copulatory plasticity in reproductive strategies may contribute more to variation in the strength and direction of sexual selection than reproductive investment in offspring.

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INTRODUCTION

Life histories of organisms are frequently shaped by trade-offs, whereby the fitness benefits of one trait (or suite of traits) are linked to deficits in another trait (Stearns, 1992). Classic examples of life-history trade-offs include whether to invest in growth or reproduction (Gadgil & Bossert, 1970), to care for offspring or court new mates (Lindström, 1998; Bjelvenmark & Forsgren, 2003) and when to reproduce relative to mortality (Zwaan *et al.*, 1995; Flatt, 2011). Because trade-offs are intrinsically linked to the determinants of fitness, optimizing the costs and benefits of traits in a particular environment is critical. Given that evolutionary trade-offs are fundamentally derived from responses to limited resources, trade-offs also shape the fitness of alternative strategies under fluctuating environmental conditions. This study considers trade-offs that may occur when mates are a limiting resource. Both male and female life histories are shaped by trade-offs between the quantity and quality of current and future reproductive opportunities. Here, it is asked whether and how females alter this investment in offspring when mates are rare *v.* common.

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Population density and mate availability fluctuate dramatically, even within individuals' lifetimes. As a result, experience with and competition for mates can lead to variation in the strength and direction of sexual selection *via* changes in pre- and post-copulatory mechanisms (Forsgren *et al.*, 1996; Hebets, 2003; Cotton *et al.*, 2006; Mück *et al.*, 2013). For example, populations shrinking under high mortality are predicted to increase investment in current reproduction, because survival and therefore future opportunities to mate are uncertain (Gadgil & Bossert, 1970; Stearns, 1992, 2000; Cichoń, 2001; Kokko & Mappes, 2005; Kokko & Rankin, 2006). Indeed, empirical evidence supports the theory of early investment in reproduction under high mortality across taxa (Reznick, 1983, 1985; Tatar & Carey, 1995; Stearns, 2000), although counter examples exist (Clutton-Brock, 1984). Populations of the two-spotted goby *Gobiusculus flavescens* (Fabricius 1779) shift from early-season male–male competition to late-season female–female competition as males become more scarce, reversing sex roles dynamically (Forsgren *et al.*, 2004; Wacker *et al.*, 2013, 2014). Similarly, the female preference for large size in this species disappears late in the season when males are rare (Borg *et al.*, 2006). Hence, male and female life histories are shaped by trade-offs that depend on both quality and quantity of current and future reproductive opportunities. Therefore, existing evidence demonstrates that fluctuation in within-season mate availability leads to changes in the strength of sexual selection and can select for phenotypically plastic responses in mating that maximize reproductive success (Stearns, 1992; Roff, 1997; Pigliucci, 2001; DeWitt & Scheiner, 2004).

Pre-mating reproductive behaviours such as mating competition and mate choice clearly respond to mate availability (Milinski & Bakker, 1992; Jirotkul, 1999; Kokko & Mappes, 2005; Kokko & Rankin, 2006; Shine *et al.*, 2006). Whether and how animals vary reproductive investment in response to mate availability, however, has received much less attention. In contrast to female choice, current reproductive investment is predicted to increase when future mating opportunities are uncertain (Gadgil & Bossert, 1970; Stearns, 1992; Cichoń, 2001; McNamara *et al.*, 2009). Because females typically invest a great deal in offspring, selection for plastic reproductive investment in response to mate availability should be strong, reflecting the potential loss of fitness if non-adaptive investments are made. Similarly, the greater the reproductive investment by males, the more exaggerated the effects should be on female reproductive success when mates are limited (Trivers, 1972; Emlen & Oring, 1977; Smith, 1977; Halliday, 1978; Patterson *et al.*, 1980; Westneat, 1988; Royer & McNeil, 1993). Indeed, there is growing evidence that mate availability influences female reproductive success (Wedell *et al.*, 2002; Smith & Reichard, 2005; Heubel *et al.*, 2008; Carrillo *et al.*, 2012), particularly in species with significant male investment in parental care (Borg *et al.*, 2002; Forsgren *et al.*, 2004; Hopwood *et al.*, 2015), or nutritional investment (Simmons & Kvarnemo, 2006; Simmons & Kotiaho, 2007; Scharf *et al.*, 2013). For example, previous work on common gobies *Pomatoschistus microps* (Krøyer 1838) has shown that female-biased sex ratios lead to larger first clutches, signifying a trade-off of increased investment early in the season at a cost of producing fewer eggs across the season (Heubel *et al.*, 2008). Although plasticity may come in more forms than previously studied, plastic reproductive investment could facilitate increased reproductive success in rapidly changing environments, for instance when populations are small, highly fragmented or experience high mortality.

Three-spined sticklebacks *Gasterosteus aculeatus* L.1758 are an established model system in sexual selection research (Rundle *et al.*, 2000; Boughman *et al.*, 2005;

Andersson & Simmons, 2006; Hendry *et al.*, 2013). They offer an opportunity to test the effects of demography on female reproductive investment in a species in which pre-mating behaviour is known to respond to altered demography (Tinghitella *et al.*, 2013). During the summer breeding season, males establish nesting territories on which to build nests and females search amongst them for mates. Under these circumstances, females, both those not yet gravid and those ready to deposit eggs, are regularly exposed to many males and nesting sites before making mating decisions (Boughman, 2006). Mating occurs throughout the breeding season in this species and occurs only within a male's nest. Males are then responsible for all parental care, which consists primarily of oxygenating eggs and defending fry.

Each time a *G. aculeatus* female develops a clutch, she has the opportunity to make a mating decision. Time is, however, limited; females ovulate all of their mature oocytes concurrently in any single clutch, thus females must invest an entire clutch with a single male and have limited time to do so (McLennan, 2006). Therefore, the rate of accepting a mate in any one encounter is not only dependent on the long-term, perceived availability of mates throughout the breeding season, but also on the short-term developmental time of the current clutch. Because body shape and size change dramatically while developing a clutch of eggs, *G. aculeatus* offer an easily observable model in which to manipulate mate availability and quantitatively measure reproductive investment.

In several post-glacial lakes in British Columbia, *G. aculeatus* demography varies within the breeding season. Adult population density decreases through the breeding season, particularly for males (J. W. Boughman, R. M. Tinghitella & M. L. Head, unpubl. data), and there is spatial variation in the operational sex ratio (OSR) across breeding sites within a given lake (Tinghitella *et al.*, 2013). As the availability of males (proportion of males) can fluctuate by over 30% from location to location in a given lake, females do indeed experience drastic differences in mate availability as they search for mates. Furthermore, female mating decisions become relaxed at the end of the season (Tinghitella *et al.*, 2013) as residual reproductive value declines (Kokko & Jennions, 2008; Lahti *et al.*, 2009), but only for females who have experienced a female-biased adult sex ratio (ASR). These females spawn more quickly and are more accepting of males, regardless of their advertised quality. Because males also must care for young, fathers are temporarily removed from the pool of potential mates, reducing availability in some areas, adding to the variation in mate availability a female experiences.

Theory predicts that sexual selection on males should be weak when a female's potential mates are rare, but this theory also ignores the possibility that reproductive investment and mating decisions may not be modified in parallel across the breeding season. If female mating decisions are relaxed when mates are rare, but females do not alter their investment in eggs or they increase current investment when the risk of not mating later in the season is high (Forsgren *et al.*, 2004), then sexual selection will be relatively weak. Lower quality males might sire a sizeable number of offspring if they wait to mate when competition is reduced, avoiding costly male competition and eventually acquiring a mating opportunity with a less choosy female. Alternatively, if investment decreases as mating decisions are relaxed, sexual selection is stronger than originally predicted: lower quality males will sire fewer offspring before the end of the season.

Theory also provides specific predictions when females must allocate whole clutches to an individual potential mate. Following the theoretical model in Heubel *et al.* (2008), it is first assumed that females can have many clutches across the season, but have

a fixed total budget of eggs. Second, it is assumed that when mates are rare, females developing clutches face uncertain future mating opportunities. Because *G. aculeatus* limnetic females typically have a single season in which to spawn, they can neither delay reproduction to the next season for better mating options nor obtain enhanced fecundity by growing to larger size (unlike other fishes; Koslow, 1996; Koons *et al.*, 2008; Secor, 2008). Thus, consistent with life-history theory, when mates are rare, reproductive effort should shift to favour investment into the first clutches or to reduce the time between clutches (Gadgil & Bossert, 1970; Stearns, 1992; Cichoń, 2001; Kokko & Mappes, 2005; Kokko & Rankin, 2006; McNamara *et al.*, 2009). This would allow females to capitalize on currently available mates and avoid the potential costs of waiting too long to spawn. Conversely, when mates are plentiful and females are more assured of future mating opportunities, investment may not shift to favour early clutches. Instead, females may produce many clutches thereby dividing out their resources among many males they will encounter across the breeding season. The partitioning of reproductive effort across clutches could increase not only the genetic diversity of offspring, as different males may be nesting at different points in the season, but also limit the amount of resource competition between siblings by temporally separating clutches.

To test whether females respond to mate availability by altering their reproductive investment, the mate availability (ASR) experienced by female *G. aculeatus* was experimentally manipulated throughout their reproductive life spans. The following were then measured: (1) the number of clutches produced over the course of the breeding season, (2) the initial clutch size (number of eggs and clutch mass) and (3) the length of time females remained gravid per clutch, across mate availability treatments.

Nearly all research to date has coupled the effects of limited mate availability with seasonal declines in mate availability. Because females encounter variation in mate availability across the breeding season, this study design teased apart different ways by which female reproductive investment may respond. The aim was to provide comprehensive knowledge of whether and how reproductive investment is plastic in response to mate availability, and how investment may modulate the strength of sexual selection in a species that responds behaviourally to mate availability.

MATERIALS AND METHODS

STUDY POPULATION AND TREATMENTS

At the beginning of the 2011 breeding season, wild limnetic *G. aculeatus* [National Center for Biotechnology Information (NCBI) Taxonomy ID: 481459] from Paxton Lake, Texada Island in British Columbia, were collected using minnow traps. *Gasterosteus aculeatus* were sexed using well-established differences in body shape (for males and females) and nuptial colour (for males) (McPhail, 1984, 1992; Hatfield, 1997) and then transferred to single sex plastic bags at equal densities that were loaded into coolers for transport by air to Michigan State University. The social conditions for both sexes were, thus, the same prior to placement in treatment tanks.

Immediately upon arrival in the laboratory, *G. aculeatus* were assigned to 283·91 (75 U.S. gallon) replicate tanks in one of the two treatments: 16 *G. aculeatus* in a 3:1 (male-biased, early-season) or 1:3 (female-biased, late-season) ratio of males to females in 12 replicate tanks (six of each treatment). These treatments represent extreme values of sex ratio variation found in the wild, particularly for the female-biased condition (Tinghitella *et al.*, 2013). Although male *G. aculeatus* are capable of raising clutches from multiple females across their lifetime,

they rarely do. Within each tank, males and females were size-matched by visual examination within sex and uniquely marked with elastomer (Northwest Marine Technology; www.nmt.us; Jones *et al.*, 2006) along the dorsal side to facilitate individual identification. After 1 day of acclimation, it was confirmed that there were no size differences between treatments or tanks in *G. aculeatus* standard length (L_S) (treatment: *t*-test: $t=0.8084$, $P>0.05$; tank: ANOVA: $F_{11,191}=0.892$, $P>0.05$) and mass (treatment: *t*-test: $t=0.9998$, $P>0.05$; tank: ANOVA: $F_{11,191}=0.103$, $P>0.05$).

Treatment tanks were visually isolated from one another using opaque, white covers applied to the outside surfaces of the tank. The artificial tank habitat included ceramic caves made of halved flower pots and plastic plants for cover. No materials were added to aid males in building nests; however, males and females could and did otherwise freely court. Makeshift nests were common, but removed to prevent females from spawning. High rates of courtship and male competition still occurred even in the absence of nests (Tinghitella *et al.*, 2013). Tanks were maintained with 14 h day lengths at *c.* 18° C, mimicking the natural conditions of their native habitat. All *G. aculeatus* were fed defrosted brine shrimp *Artemia* sp. and bloodworms *Chironomus* sp. once per day *ad libitum*.

TRACKING DAILY FEMALE REPRODUCTIVE INVESTMENT

To assess daily changes in female reproductive status, each female was visually assessed on a 0–5 gravidity scale, where 0 indicated a non-gravid female and 5 indicated a female ready to release her clutch (as evidenced by an open genital pore and a plump, swollen abdomen; Frommen *et al.*, 2012). A gravidity score for each female was recorded blind to the previous day's measurement. This allowed observers to calculate the number of days a given female was gravid, as well as the total number of gravid females per tank each day. Visual assessment of females on the 0–5 gravidity scale was performed by a single researcher. Additionally, changes from a gravidity score of 5 on one day to 0 the following day indicated release of a clutch (either because the female dropped the clutch naturally or because the clutch was extracted as described below), and allowed calculation of the total number of clutches each female developed across the season. These in-tank observations minimized the amount of handling of the *G. aculeatus*.

TRACKING REPRODUCTIVE INVESTMENT IN INITIAL CLUTCHES

All four females in male-biased tanks and a randomly determined sub-set of four females from female-biased tanks were identified as focal individuals ($n=48$) and used to obtain additional body size and clutch measurements. When these females attained a score of 5 on the gravidity scale, they were weighed to the nearest hundredth of a gram on an OHAUS Scout PRO SPE 202 balance (<http://us.ohaus.com/en/home/products/product-families/SP-US.aspx/>). To obtain L_S to the nearest 0.01 mm, females were photographed using a Canon G-15 digital camera (www.usa.canon.com/cusa/support/consumer/digital_cameras/powershot_g_series/powershot_g15) and the distance between digital landmarks at two extremes of the body (the anterior tip of upper lip and caudal border of the hypural plate at the lateral midline) was calculated using the programme Past (<http://folk.uio.no/ohammer/past/>) according to the established methods (Taylor *et al.*, 2006; Cooper *et al.*, 2011). The female was then gently squeezed to extract her clutch. The clutch was weighed, the number of eggs counted and the female was reweighed post-extraction. Because previous work (Heubel *et al.*, 2008; Carrillo *et al.*, 2012) suggests first clutches are mostly altered by sex ratio differences, and animal handling and egg extraction could decrease survivorship and reclutching rate (one of the key variables under study), extraction of eggs was conducted only for a female's initial clutch to minimize adverse effects on *G. aculeatus* health and sample size. Note that *t*-tests conducted following the experiment showed no effect of handling on reproduction (when comparing the four focal females to the other females within each female-biased tank; all $P>0.05$).

STATISTICAL ANALYSIS

Because the reproductive investment of multiple females from each replicate tank was assessed, mixed effect model analysis was appropriate to evaluate how perceived availability of mates affects female reproductive investment. Measures of reproductive investment across mate availability treatments included the number of clutches a female had throughout the breeding season, the number of eggs per initial clutch, initial clutch mass, the number of days spent either non-gravid (stage 0), developing eggs (stages 1–4) and fully gravid (stage 5) during the season and finally the start and stop of seasonal reproduction. Mixed models were conducted within R (R Core Team; www.r-project.org) using the lme4 library (Bates *et al.*, 2013). Treatment (male or female bias) was entered as a fixed effect into all models. Intercepts for females (nested within replicate treatment tanks) and number of clutches (for models concerning days gravid) were included as random effects. To account for covariances in daily measures of gravidity, repeated measures were incorporated into models measuring days gravid. Plots of residuals for each response variable were visually inspected to detect deviations from homoscedasticity or normality, and generalized linear mixed models were used with a Poisson distribution when appropriate. The fit of the full model (with the fixed treatment effect) was compared against a reduced null model without the fixed effect (only random effects) to test whether the fit of the model decreased significantly ($P < 0.05$) using χ^2 tests (Winter, 2013). Finally, Levene's test was used to test for significant difference in variances between treatments. Data are presented as means \pm S.D.

RESULTS

During the summer breeding season of this study, females under both male and female-biased conditions had an equal likelihood of being reproductive (producing at least one clutch) ($\chi^2 = 0.112$, d.f. = 2, $P > 0.05$). Females in both mate availability treatments had 1.188 ± 1.149 clutches (range = 0–4). As the focus was on whether and how reproductive investment changed in response to mate availability, for the following analyses, females who were never reproductive ($n = 8$) were removed.

Females in male-biased tanks produced 1.47 times the number of clutches produced in female-biased tanks. Despite differences in the number of clutches produced, due to greater variance within treatments, females from both treatments generated an equal number of clutches across the season and the variance did not differ between treatments ($\chi^2 = 2.148$, d.f. = 2, $P > 0.05$, Levene's test $F = 0.002$, $P > 0.05$). Reproductive females (those which had at least one clutch) had 1.970 ± 0.825 clutches across the season, regardless of treatment, and further examinations of first clutch mass and first clutch egg number also revealed no difference in investment across treatments (first clutch mass: $\chi^2 = 0.033$, d.f. = 1, $P \geq 0.05$, Levene's test $F = 0.215$, $P \geq 0.05$ and egg number: $\chi^2 = 0.033$, d.f. = 1, $P > 0.05$, Levene's test $F = 1.027$, $P \geq 0.05$; see Fig. 1).

Next, the timing of reproductive investment was assessed across mate availability treatments. Variables measured included the number of days females spent in a completely non-gravid state (0 on the gravidity scale) both for consecutive days (spans) of non-gravidity (time between clutches, or the interclutch interval) and the total days spent in a non-gravid state across the season. This method revealed how clutches are distributed within a season and also captured differences in potential failed clutches which may not have developed fully. Interestingly, most days of the reproductive season were spent in a non-gravid (0) state (101.880 ± 12.248 days). Females spent 12.755 ± 9.352 days between 0 and other states of gravidity (the interclutch interval). When considering differences between mate availability treatments, there was neither

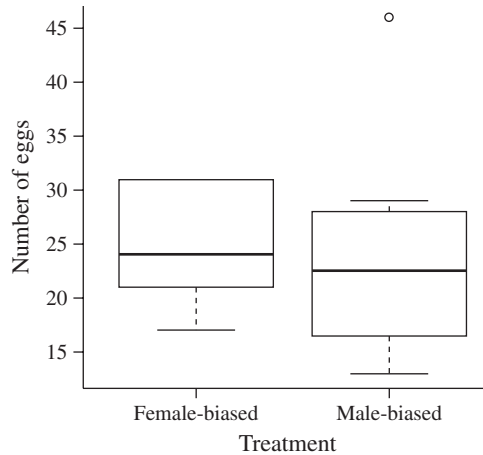


FIG. 1. Comparison of the number of eggs per initial clutch between *Gasterosteus aculeatus* female and male-biased mate availability treatments. The \square represent values from the lower to upper quartile (25–75 percentile; first and third quantiles) and are intended to give c. 95% c.i. for differences in the two data sets. — represents the median and extreme values are represented by \circ .

a difference in the total number of days spent in a non-gravid state (Fig. 2; $\chi^2 = 0.967$, d.f. = 1, $P > 0.05$, Levene's test $F = 0.188$, $P > 0.05$), nor in interclutch interval length (Fig. 3; $\chi^2 = 1.288$, d.f. = 1, $P > 0.05$, Levene's test $F = 0.314$, $P > 0.05$). This indicates that females spent little time developing clutches across the season, with the majority of the days spent in a non-gravid state.

Next, the number of days a female spent developing a given clutch of eggs, before she was ready to release the clutch (stages 1–4), was examined. The consecutive number

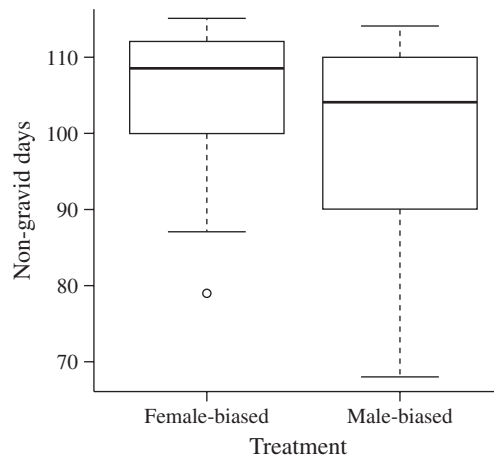


FIG. 2. Comparison of the number of days spent in a non-gravid state between *Gasterosteus aculeatus* female and male-biased mate availability treatments. The \square represent values from the lower to upper quartile (25–75 percentile; first and third quantiles) and are intended to give c. 95% c.i. for differences in the two data sets. — represents the median and extreme values are represented by \circ .

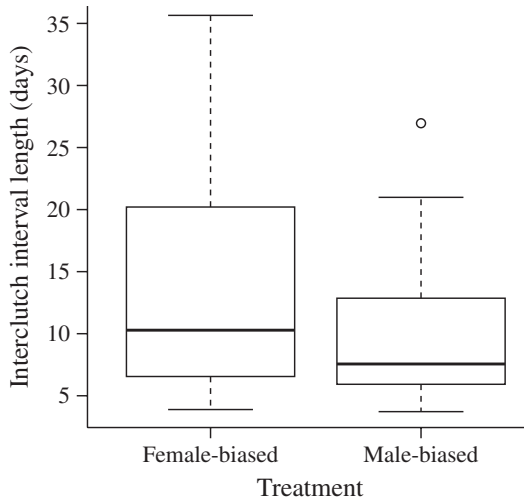


FIG. 3. Comparison of the interclutch interval length (the number of consecutive days a female *Gasterosteus aculeatus* is completely non-gravid, *i.e.* neither carrying nor developing a clutch) between female- and male-biased mate availability treatments. The \square represent values from the lower to upper quartile (25–75 percentile; first and third quartiles) and are intended to give *c.* 95% C.I. for differences in the two data sets. — represents the median and extreme values are represented by \circ .

of days on which the gravidity score ranged from 1 to 4 was the measure of time spent developing a clutch. A drop to 0 gravidity before reaching a fully gravid state (5) was considered a lost clutch. The treatments did not differ significantly ($\chi^2 = 0.076$, d.f. = 1, $P > 0.05$, Levene's test $F = 1.067$, $P > 0.05$); females spent an average of 9.690 ± 1.859 days of the season in a state of clutch development, and each span of development was relatively quick, lasting only 2.490 ± 0.212 days.

Then, the number of days spent in a completely gravid state (5 on the gravidity scale) during the 116 day experiment was assessed across sex ratio treatments. Females under both male and female-biased conditions spent 3.55 ± 0.64 days across the season in a completely gravid (5) state ($\chi^2 = 1.955$, d.f. = 1, $P > 0.05$, Levene's test $F = 0.499$, $P > 0.05$), and each span of gravidity lasted < 2 days (1.670 ± 0.181 days; $\chi^2 = 0.331$, d.f. = 1, $P > 0.05$).

Finally, the time to first reproductive day (calculated from the day treatments were established) and time to last reproductive day (the end of reproduction for each female) were assessed. Given the importance of diet and time necessary to develop a clutch, females may be physiologically constrained from modulating clutch numbers, size or time to development; however, when during the season a female begins to reproduce may be more plastic, and the variation in this starting point may facilitate females' ability to capitalize on short-term variation in available mates. Therefore, Julian dates were calculated to determine the first day on which each female began developing a clutch (score > 0 on gravidity scale) as a measure of the beginning of reproduction for each female. Females, regardless of mate availability, started reproduction in early May and stopped reproduction at the end of July with *c.* 1 week of variance during this breeding season experiment ($\chi^2 = 0.736$, d.f. = 1, $P > 0.05$, Levene's test $F = 0.209$, $P > 0.05$ and $\chi^2 = 0.722$, d.f. = 1, $P > 0.05$,

Levene's test $F = 1.563$, $P > 0.05$, respectively); *i.e.* mate availability in female *G. aculeatus* does not appear to determine how early or late in the season a female is likely to become reproductive.

DISCUSSION

Both male and female life histories are shaped by trade-offs between the quality and quantity of current and prospective mates. This experiment investigated whether and how female reproductive investment responds to mate availability, and whether those responses are modified in parallel with known patterns of mate choice. The results show that limited plasticity in reproductive investment may keep females from mitigating the effects of relaxed choosiness when mates are rare. Indeed, there was no evidence that the number of reproductive females, clutches per season (per female), number or mass of eggs in initial clutches or the timing of reproduction differed between mate availability treatments.

When males are rare and time is short (females are approaching the end of their reproductive life spans), female *G. aculeatus* relax their mating decisions (Tinghitella *et al.*, 2013). As female mating decisions are relaxed under low mate availability, but reproductive investment appears fairly canalized, sexual selection should be relatively weak under these conditions. Because females deposit an entire clutch in the nest of a single male when spawning, all chosen males, regardless of quality, can potentially receive the same reproductive investment because there is limited plasticity in how females allocate reproductive resources. In particular, if female choice is relaxed, allowing lower quality males to spawn, males of lower quality could enjoy the most reproductive success by avoiding costly male competition (particularly at higher densities) through either (1) nesting in less dense areas or (2) waiting to nest until other males are providing parental care to eventually acquire a mating opportunity once males become rare. Thus, less preferred males could reap more benefits simply by being available in the right place (where there are fewer males) and at the right time (when females are ready to spawn).

Plasticity in reproductive investment may also be limited by costs such as predation risk and incomplete information about search costs. Specifically, spending more days fully gravid per clutch, or having more clutches, can increase a female's risk of predation (Magnhagen, 1991). Because females have incomplete information about the probability of success in continued mate search (Stephens & Krebs, 1987; Real, 1990; Wiegmann *et al.*, 2010), they experience costs associated with risk (Raiffa, 1970; Trimmer *et al.*, 2011). Risks should increase with time unmated (as eggs may become unviable), rarity of mates and prevalence of predators. Although having developed eggs prepares a female should she encounter a rare mate, the risks of unviable eggs and expansive searches could counter selection for plasticity in increasing the expected duration of gravidity per clutch.

Based on observations of short, repeated visits by females to male territories, Dale & Slagsvold (1996) suggested that the number of times a male is encountered on a territory might be an informative signal for females about future mating opportunities, and therefore the risk of continued search (Raiffa, 1970; Trimmer *et al.*, 2011). Males within this study, however, were not allowed to maintain complete nests in their treatment tanks to prevent females from depositing eggs in them (because females' mating

decisions were simultaneously being assessed in a parallel experiment using no-choice mating trials with nesting males; Tinghitella *et al.*, 2013). Experience with both males and their nests may be necessary for females to assess and respond to differences in mate availability by adjusting reproductive investments; thus, from the perspective of searching females, although there was a difference in the ASR between treatments, the OSR could have been strongly female biased in both treatments. Given that the experimental set-up for the study used the same individuals and actually did find an effect of sex ratio treatment on female mating decisions (Tinghitella *et al.*, 2013), it is likely that the experimental design was sufficient to produce an effect on female reproductive investment, if one exists. Similarly, had females within a treatment tank been simultaneously gravid often, the OSRs could have been more variable than the ASRs would suggest. Multiple fully gravid females, however, scarcely occurred within a tank at the same time, thus the sex ratio remained largely consistent for the duration of the experiment.

Although numerous examples exist demonstrating female plasticity in investment in response to her environment, including in response to predation (Giesing *et al.*, 2011), conspecific brood parasitism (Lyon, 1998) and attractiveness or parenting ability of males (Sheldon, 2000; Kolm, 2001; Stiver & Alonzo, 2009; Kindsvater *et al.*, 2013; Poisbleau *et al.*, 2013; Soma & Okanoya, 2013), there is limited evidence to suggest plasticity in female reproductive investment due to demography. Perhaps, the notable exception of plasticity in reproductive investment shown by Heubel *et al.* (2008) is facilitated by relatively extreme within-season variation in sex ratios in *P. microps*. This variation is substantially more dramatic than that observed in *G. aculeatus*, and thus the selection pressure favouring the evolution of plastic reproductive investment may simply be weak. The difference in effects between these species may be exaggerated by the manner in which clutches are partitioned between males. Female *G. aculeatus* must deposit all of their clutches in the nest of a single male, thus they must make investment decisions earlier with uncertainty as to whether any mating will be secured for the clutch under development. *Pomatoschistus microps*, in contrast, could secure a mating with a portion of a clutch and then delay to deposit the remaining eggs with another male. Thus, selection pressures on reproductive investment may differ in these species depending on clutch size, clutch partitioning and the timing of mate encounters.

Likewise, limitations in observed plasticity may also be due to energetic constraints. Despite ample food, females in this study generally had few clutches, although clutch totals were within range of the expected reproductive output across populations of *G. aculeatus* (Wootton, 1976). Therefore, differences between clutches, particularly in timing, may be difficult to examine. Adding to this the fact that females are only 30% physiologically efficient in producing eggs (*i.e.* resources are not very efficiently converted to eggs; Wootton & Evans, 1976), specific reproductive investment trade-offs and reallocations of energy to other functions would need to be quite large to detectably alter days gravid, clutch sizes, clutch number or egg number.

Female *G. aculeatus* could also plastically adjust reproductive investment in response to mate availability in other ways than measured in this experiment. For instance, females could alter investment in offspring by varying their quality, not quantity, through increasing cortisol levels and egg size to influence offspring survival (Giesing *et al.*, 2011). Egg size was not measured here, but increasing egg size is known to positively affect feeding (Knutsen & Tilseth, 1985), swimming abilities (Ojanguren *et al.*, 1996) and survival at both egg and larval stages (Lillelund & Lasker,

1971; Henrich, 1988; Leggett & Deblois, 1994), all of which may translate into fitness advantages in their current environment.

Given that *G. aculeatus* are iteroparous but unlikely to live to a second breeding season, deferring reproduction is probably disfavoured evolutionarily in this species, particularly for females experiencing an excess of mates (male-biased conditions). A more probable strategy might be to modify the final clutch (*e.g.* in mass, egg number and egg size), maximizing the terminal investment (Clutton-Brock, 1984). This experiment did not capture variation in terminal investment, although characteristics of final clutches were not investigated because of the risk involved in extracting clutches.

This study highlights the importance of the interplay between mate availability, female mate choice and female reproductive investment shaping variation in the strength and direction of sexual selection. Laboratory studies aimed towards estimating investment and behavioural interactions will probably benefit from considering that the demographic effects of mate choice (intersexual selection) and mate competition (intrasexual selection) for both males and females may be tempered by the degree of plasticity in female investment. As Moura & Peixoto (2013) point out in their meta-analysis, responses of females and males may differ from species to species as sex ratios become more male biased; these responses may be exaggerated in species where males provide care. Recent evidence on burying beetles *Nicrophorus vespilloides* shows increased care under increased reproductive competition (Hopwood *et al.*, 2015), but more evidence is necessary to determine the extent to which this pattern exists across species, and whether it is exaggerated or ameliorated by other alterations in investment. Thus, future work should expand understanding of how demography influences reproductive strategies of males, in response to mate competition and female choice, particularly in species where males provide care.

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