When should male squid prudently invest sperm?

Amy K. Hooper [a, b, *], Benjamin J. Wegener [a], Bob B. M. Wong [a]

[a] School of Biological Sciences, Monash University, Victoria, Australia
[b] Evolution and Ecology Research Centre, School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, Australia

Ejaculate production can be costly and males are expected to prudently allocate this potentially limiting resource to higher quality females. However, relatively little is known about facultative sperm allocation in response to the quality distribution of sequentially encountered females, despite this being a more realistic scenario for males in many species. Here, we examined patterns of male investment in a squid, *Sepiadarium austrinum*, when presented sequentially with small versus large females. Owing to a positive size — fecundity relationship in this species, large and small females are expected to differ in terms of their perceived quality to males as potential mating partners. Yet, despite large sperm investment and significant variation in female quality, sperm investment was determined only by mating order, with males consistently decreasing sperm investment in second matings. These results highlight that, when mates are encountered sequentially rather than simultaneously, prudent sperm allocation may not occur when it is otherwise predicted.
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Although females have traditionally been considered the choosy sex, patterns of male mate choice are now well established (Bonduriansky, 2001). In particular, males are expected to be choosy when females vary in quality or males invest significantly in matings (Bonduriansky, 2001; Kokko & Johnstone, 2002). Classic examples of high male investment in matings include species in which males engage in parental care (Gwynne & Simmons, 1990), provide nuptial gifts (Kvarnemo & Simmons, 1998; Tigreros, Mowery, & Lewis, 2014) or engage in extensive mate guarding (Johnson & Hubbell, 1984). For males of many species, significant costs of ejaculate production can make sperm a potentially limiting resource (Dewsbury, 1982). This can occur when males produce large ejaculates (Gwynne, 1981) or complex seminal proteins (Cornwallis & O'Connor, 2009). When this occurs, males may benefit from preferentially allocating this potentially limiting resource to higher quality females, and, in fact, evidence of prudent sperm allocation is now well documented (Kelly & Jennions, 2011). Additionally, some males become choosier as they become increasingly sperm depleted, as has been shown in *Drosophila* (Byrne & Rice, 2006). However, maximizing reproductive output is often more complex than determining which mating opportunities are better than others. Often, males have no prior information regarding quality distribution of females prior to encountering their first potential mate, and furthermore have limited opportunities to directly compare the quality of potential mates (Janetos, 1980; Jennions & Petrie, 1997). For example, where males maintain territories, or individuals are sparsely distributed in the environment, prospective mates will rarely be encountered at the same time (Forbes, Boates, McNeil, & Brison, 1996; Wong & Svensson, 2009). Opportunities for simultaneous comparison of mates are therefore likely to be confined to mating systems in which mate search costs are low, such as in highly social species and lek mating systems where individuals congregate together (Jennions & Petrie, 1997). Consequently, sequential encounters of mates are expected to be a much more common (and biologically realistic) scenario for most species. This raises some important questions regarding mate choice theory, such as how individuals are then able to compare mates of different quality, and how individuals make decisions about which potential mates to invest in when there is uncertainty regarding future mating opportunities (Janetos, 1980; Real, 1990). However, surprisingly few studies have investigated these patterns (Gibson & Langen, 1996; Jennions & Petrie, 1997).

Studies of male courtship have shown that males of several species can strategically adjust their courtship effort towards females of varying quality when they are encountered sequentially (Bateman & Fleming, 2006; Reading & Backwell, 2007; Wong & Svensson, 2009). For example, male desert gobies, *Chlamydogobius eremius*, decreased courtship effort in small females when they

* Correspondence: A. K. Hooper, Evolution and Ecology Research Centre, School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, 2052, Australia.
E-mail address: amy.hooper@student.unsw.edu.au (A. K. Hooper).

had previously been presented with a larger female (Wong & Svensson, 2009). Similarly, naïve male field crickets, *Gryllus bimaculatus*, did not adjust courtship effort in response to female size, but did so in subsequent matings, suggesting that males might only become choosy after securing a mating with at least one female (Bateman & Fleming, 2006). Relatively few studies, by contrast, have investigated how males might similarly adjust sperm investment over multiple sequential matings (Engqvist & Sauer, 2001; Ramm & Stockley, 2014). One exception was a study in mice, *Mus musculus domesticus*, which found that males adjusted sperm allocation to females in response to sperm competition risk when females were encountered sequentially; however, investment was not affected by mating order (Ramm & Stockley, 2014). It remains to be tested, however, whether these forms of strategic sperm investment are prevalent in other species in which sperm is limiting.

The southern bottletail squid, *Sepiadarium austrinum*, presents an ideal opportunity to investigate strategic sperm investment patterns. Male bottletail squid transfer sperm in prepackaged bundles (spermatophores), transferring several to females during copulation. This mode of sperm transfer allows for relatively easy measurement of male reproductive investment, as spermatophores can be collected after copulation. Males invest heavily in ejaculates, depleting up to 60% of their available sperm stocks during a single copulation (Wegener, 2011). As females store spermatophores in the buccal cavity (an invagination around the mouth; Norman, 2003), spermatophores are likely to be vulnerable to both consumption by females (Wegener, Stuart-Fox, Norman, & Wong, 2013b) and removal by rival males. Males are also less likely to attempt matings with smaller females, which are more likely to consume large proportions of spermatophores after mating (Wegener et al., 2013b). Accordingly, sperm investment in this species is a costly endeavour with potentially low payoffs. Hence we might expect males to strategically invest sperm in higher quality females. Males may also become increasingly prudent after mating when their sperm stocks are depleted (Byrne & Rice, 2006). To test this, we presented males sequentially with two females of different size, and subsequently measured mating investment. Furthermore, as males have been shown to preferentially mate with larger females (Wegener et al., 2013b), and as such body size is assumed to be an indicator of female quality, we investigated the size − fecundity relationship in females of this species.

METHODS

Collection and Housing

Juvenile squid were collected by SCUBA from Port Philip Bay near Queenscliff (38°10′12″S, 144°43′8″E), Australia, in May and June 2011. Animals were then housed in continuous flow sea water tanks under natural light and temperature conditions. Females were housed in 9-litre communal holding tanks in groups of five to eight, and males were housed individually in 2.3-litre tanks. All animals were fed a diet of live amphipods and *Palaemonetes* shrimp ad libitum. As the bottletail squid is an annual species, with all juveniles hatching at approximately the same time each year, all experimental animals were assumed to be of a similar age at the time of experimental assays.

Experimental Procedure

Males were haphazardly allocated to one of two treatments: males were either presented sequentially with a large (4.58 ± 0.43 g) then a small (3.04 ± 0.38 g) female ($N = 15$) or a small then a large female ($N = 15$). The upper and lower quartiles of weight distribution were used to define large and small

females, and there was a significant difference between these size classes (Welch two-sample *t* test: $t_{27.9} = 14.57$, $P < 0.001$). Males were randomly allocated to treatments, and there was no difference in male size between treatment groups (1.74 ± 0.29 g; Welch two-sample *t* test: $t_{57.85} = -0.67$, $P = 0.501$). To initiate matings, the first female was introduced to the centre of the male's holding tank. After mating, this female was removed and males were left to recuperate for 30 min before the second pairing was initiated. This length of time was chosen to simulate a high mating rate, so males would not have the opportunity to replenish sperm reserves before the second mating, yet still give males a small break between female presentations to rest to increase the chance they would mate again. Immediately after mating, females were humanely euthanized in 5% $MgCl_2$ sea water solution, weighed, and the buccal cavity and stomach dissected to determine the number of spermatophores received during mating. The mantle cavity was also dissected to determine the number of mature eggs present at the time of mating. Focal males were similarly euthanized after the second mating, and their spermatophoric organ dissected to determine how many spermatophores remained. As male bottletail squid take several days to replenish spermatophore reserves (Wegener, 2011), this enabled us to calculate the number of spermatophores present at the beginning of trials by adding the number of spermatophores remaining and the total number transferred to females. Latency to mate and duration of mating were measured as further indications of male reproductive effort.

Sperm Quality Analysis

Five spermatophores received by each female were randomly selected and their contents analysed for sperm quality. Spermatophores were gently crushed to release their contents in 1 ml of sea water, and the number of sperm cells present in the resultant solution was determined using a haemocytometer. Sperm viability was assessed with a dual-fluorochrome vital dye (SYBR-14 and propidium iodide, Molecular Probes Inc., Eugene, OR, U.S.A.) to determine a live/dead cell ratio, following a procedure adjusted from Sherman, Uller, Wapstra, and Olsson (2008). This involved mixing 200 µl of the sperm/sea water solution with 2 µl of 1:50 diluted (with HEPES buffer) SYBR-14 dye and 2 µl of propidium iodide, and left to incubate at room temperature in the dark for at least 20 min. Differentially stained sperm cells were then examined using fluorescent microscopy (blue-red-green filter 450−600 nm wavelength) at 400× magnification. Viability was measured by calculating the ratio of cells that were alive (stained green) to those that were dead (stained red), when at least 500 cells were counted from multiple random fields of view. No moribund sperm were observed.

Statistical Analysis

Patterns of male investment were analysed using linear mixed-effects models. Female size class (large or small) and presentation order were entered as predictor variables (fixed factors), with male ID entered as a random factor. Dependent variables analysed were: latency to first mating attempt, duration of mating, total number of spermatophores invested in females, mean number of sperm cells per spermatophore transferred and viability of sperm. Latency and duration of mating were log transformed prior to analysis where this improved normality of residual errors. Means are presented as ±1 SE. Female fecundity was analysed using a simple linear regression of the number of mature eggs stocked as a function of female weight at the time of mating.

Ethical Note

This research complies with all relevant State and Federal laws of Australia. All procedures were approved by Monash University Ethics committee (ethics permit number BSCI/2009/06). During all procedures, care was taken to reduce handling time to minimize stress of the animals. For the euthanasia, the concentration of $MgCl_2$ in the sea water solution was increased from 2% to 5% over 30 min so that animals were anaesthetized prior to increasing concentrations to a lethal level (Messenger, Nixon, & Ryan, 1985).

RESULTS

We found that latency to mate was unaffected by female size (Table 1). Males were faster in their attempts to mate with the second female presented to them, taking, on average, 10.9 ± 2.8 s, compared to 19.4 ± 6.2 s with the first female. On average, mating duration was 228.5 ± 26.49 s, which was not correlated with either the number of spermatophores transferred by males ($F_{1, 29} = 0.10$, $P = 0.750$), female size or mating order (Table 1).

On average, the number of sperm cells contained in each spermatophore was $2.03 \times 10^6 \pm 4.83 \times 10^4$, and this was unaffected by either female size or mating order (Table 1). However, mating order significantly affected spermatophore investment, with males consistently transferring fewer spermatophores during second matings (Fig. 1, Table 1). The mean number of spermatophores present in males at the beginning of mating trials was 61.90 ± 2.8, with only 31.1 ± 1.1% of original stocks remaining after two matings. Across all matings the average sperm viability recorded was 29.89 ± 1.45% cells alive. Viability of sperm transferred to females was not influenced by mating order or female quality (Table 1). No moribund cells were observed.

We found a significant positive relationship between female weight and number of eggs carried (linear regression: $r^2 = 0.62$, $F_{1, 58} = 95.48$, $P < 0.001$; Fig. 2).

DISCUSSION

We found no evidence of strategic sperm allocation to larger females in southern bottletail squid, despite evidence that males would gain significant reproductive benefits from prudent male choice. First, the positive size − fecundity relationship we found indicates that females vary significantly in quality, and that larger females represent a higher reproductive value. Second, males become highly sperm depleted after only two matings, indicating
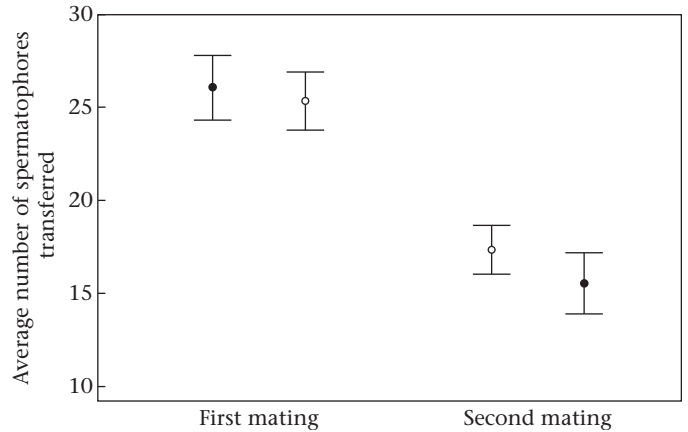

Figure 1. Average ± SE number of spermatophores transferred to females in sequential mating trials in southern bottletail squid, *Sepiadarium austrinum*, where males were mated with a small then a large ($N = 15$) or a large then a small female ($N = 15$). Filled circles represent large females; open circles represent small females.

that this sperm investment reduces the number of subsequent matings in which a male can invest. Theory therefore predicts that males should be selected to optimally allocate limited mating resources (ejaculate) to higher quality females; yet, despite these factors, mating investment was determined only by mating order.

We hypothesize that low mating rates in the wild may overcome benefits of prudent male choice in this species. Theory predicts that males should be choosy when reproductive investment decreases the number of subsequent matings in which a male can invest (Bondurianksy, 2001). Male bottletail squid produce a mean of 4.6 ± 2.9 new spermatophores per day (Wegener, 2011); hence the average investment found in this study, as well as the consistent decrease in investment in second matings (Fig. 1), suggests that sperm is a limiting resource for males of this species. However, if mating opportunities are rare, it may be more beneficial for males to invest in matings indiscriminately, as shown here, rather than selectively allocate sperm to females, despite significant variation in female quality (Barry & Kokko, 2010; Head, Jacomb, Vega-Trejo, & Jennions, 2015; Reading & Backwell, 2007). Previous studies on fiddler crabs, *Uca mjoebergi* (Reading & Backwell, 2007) and salamanders, *Desmognathus santeetlah* (Verrell, 1995) directly compared male choice under sequential and simultaneous choice scenarios, and found that, although males showed a preference for larger females when mate encounter rates were high, they did not

Table 1
Linear mixed-effects models of male reproductive investment in southern bottletail squid, *Sepiadarium austrinum*

Dependent variable	Predictor variable	F_{df}	P
Latency to first mating attempt	Female size class	$0.09_{1, 27}$	0.761
	Mating order	$4.86_{1, 27}$	**0.036**
	Interaction	$4.03_{1, 27}$	0.055
Duration of mating	Female size class	$0.27_{1, 27}$	0.610
	Mating order	$0.02_{1, 27}$	0.881
	Interaction	$0.68_{1, 27}$	0.428
Total number of spermatophores invested in females	Female size class	$0.19_{1, 27}$	0.665
	Mating order	$57.74_{1, 27}$	**<0.001**
	Interaction	$0.46_{1, 28}$	0.505
Mean number of sperm cells per spermatophore	Female size class	$0.11_{1, 27}$	0.742
	Mating order	$2.08_{1, 26}$	0.161
	Interaction	$0.10_{1, 26}$	0.756
Sperm viability	Female size class	$0.00_{1, 26}$	0.999
	Mating order	$0.08_{1, 26}$	0.775
	Interaction	$0.40_{1, 26}$	0.534

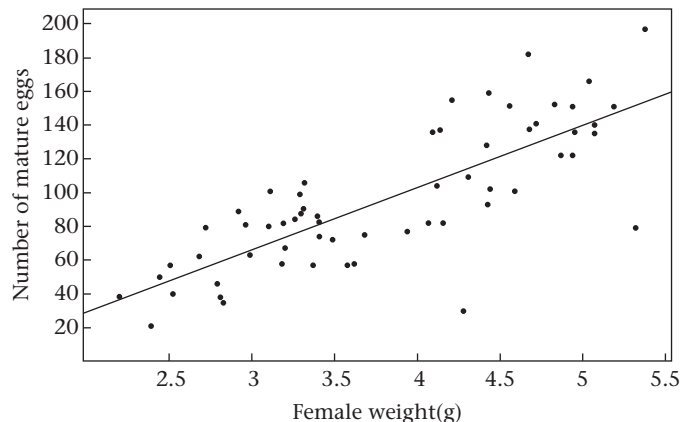Significant P values are shown in bold.

Figure 2. Relationship between female weight and number of mature eggs stocked in southern bottletail squid, *Sepiadarium austrinum*, with line of best fit.

show any preference when sequentially presented with females. These studies demonstrate that, in mating systems in which mates are encountered sequentially, benefits of prudent allocation of mating resources may be decreased because mating opportunities are necessarily lower and search costs are higher (Barry & Kokko, 2010). The consistent decrease in sperm investment in second matings we observed, as well as the significant time required time to produce new spermatophores, may indicate that natural mating rates are low in bottletail squid (Reinhold, Kurtz, & Engqvist, 2002), and therefore there may be weak selection for males to be choosy even when they are sperm depleted from previous matings. Although adult bottletail squid occur in relatively high densities (up to 5 adults/m^2; A. K. Hooper, B. J. Wegener & B. B. M. Wong, personal observation), mating rates in the wild are unknown. None the less, in species in which individuals congregate socially or in leks, male mating success can be highly variable, with many males experiencing zero reproductive success (Gibson & Bradbury, 1985; Howard, 1979). In these species, male mating success is often determined by female choice or male competition for access to females, which may be prevalent in bottletail squid also. However, effects of these factors on male choice in this species remain unknown.

Although males did not preferentially allocate sperm to larger females, there may be other female quality cues that are more important in this species. We found that larger female bottletail squid carry more eggs by over an order of magnitude (Fig. 2), which strongly suggests that larger females are more fecund. However, the size − fecundity relationship in squid may be more complex than just number of eggs stocked at any time, and this may not necessarily translate into increased lifetime reproductive output or increased clutch size (Maxwell & Hanlon, 2000). Therefore female quality may be related to other traits in this species. For example, in many species, female mating status is the best indicator of female quality (Gaskett, Herberstein, Downes, & Elgar, 2004; Zahradnik, Lemay, & Boulding, 2008). Female bottletail squid can store spermatophores for up to 21 days (Wegener, Stuart-Fox, Norman, & Wong, 2013a), during which time females are likely to mate with multiple males, as is common among cephalopods (Hanlon & Messenger, 1999). Females store spermatophores in the buccal cavity, and invagination around the mouth, which is also the site of fertilization. Therefore they are exposed to rival males as well as the female's external environment. Hence male mating behaviour is likely to be influenced by the risk of sperm competition, as well as the female's likely future mating behaviour, as was shown in a recent study of sequential mating investment in wild mice (Ramm & Stockley, 2014). If this is the case, males in our study may be responding to all females as high-quality (virgin) females. Furthermore, high sperm investment and large testis size (approximately 25–30% of body cavity; Wegener, 2011) may also indicate high levels of sperm competition in this species (Parker, Ball, Stockley, & Gage, 1997; Parker & Birkhead, 1998). Further study in this species regarding the dynamics of female mating status, sperm competition and male choice is needed.

In conclusion, despite potential benefits of prudently allocating sperm to larger females, we found no evidence of this in southern bottletail squid. Hence, our results demonstrate that the prevalence of sequential choice may overcome benefits of prudent sperm allocation strategies where mating is costly and male choice is otherwise predicted.

Acknowledgments

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