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Nocturnal Mating Behaviour and Dynamic Male Investment of Copulation Time in the Southern Blue-Ringed Octopus, *Hapalochlaena maculosa* (Cephalopoda: Octopodidae)

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ABSTRACT

The southern blue-ringed octopus**,** *Hapalochlaena maculosa* (Hoyle, 1883) is a nocturnal species that exhibits a mating system in which females hold sperm from multiple males over a one to two month breeding window before laying a single egg clutch. Contrary to most studied animal mating systems where anisogamy exists, gamete package production is limited for both males and females of this species ($\sim$50 spermatophores/eggs). This presents an animal model for studying aspects of sperm competition and dynamic mate choice behaviours. The present study reports on the mating behaviour of *H. maculosa* observed under laboratory conditions using infrared closed-circuit television video footage. Rates of male copulation attempts increased with male size, while female receptivity to mating attempts increased with female size, resulting in larger animals of both sexes gaining more copulations and spending more time per day in copulation. There was some evidence of female preference of larger males, but no male preference of females based on measured morphological traits. Both sexes terminated copulations in equal frequencies but male-terminated copulations were significantly shorter in duration. Males were more likely to terminate copulation early with females they had previously mated with, however were less likely to do so if the female had recently mated with a different male. Among male-terminated copulations, males mated for longer with females that had previously mated with other males in the trial. Male-male mounts were as common as male-female mounts, suggesting that male *H. maculosa* are not able to discriminate the sex of conspecifics. These findings suggest male strategic allocation of spermatophores based female mating history is an important factor influencing mating behaviours of this species.

ADDITIONAL KEYWORDS: mate choice - *Octopus* – operational sex ratio – sperm competition.

INTRODUCTION

Sexual selection is a form of intra-specific competition in which differential reproductive success within one or both sexes in a species can lead to the evolution of phenotypic traits and/or behaviours that aid individuals to increase their own level of reproductive success (Darwin, 1906; Bateson, 1983). To date, the processes of sexual selection have been predominantly studied within vertebrate and insect mating systems (West-Eberhard, 1983; Andersson & Simmons, 2006). Within these animal models, anisogamy, which is the differential investment between males and females towards their gametes, leads to the reproductive success of most females of these taxa to be limited by the resources they have access to, and male reproductive success to be primarily limited by the numbers of females they can successfully mate with (Kodric-Brown & Brown, 1987). Therefore, where anisogamy exists, sexual selection typically imposes females to selectively mate with higher quality and/or genetically compatible males, and males to evolve traits or behaviours that enable them to achieve more copulations with a higher number of females, and to attain greater fertilisation success with the females they mate with (Darwin, 1906; Bateson, 1983; Kodric-Brown & Brown, 1987).

Cephalopods (Mollusca: Cephalopoda) present a different style of mating system from many other taxa, especially vertebrates, because male mate choice might be a critical factor influencing reproductive behaviours and spawning patterns within this class of animals. The fact that spermatozoa are encased in a finite number of discrete spermatophores, one or more of which are transferred to the female during copulation, imposes a disparity in which male

spermatophores may be as limited or more limited than female eggs (Mann, 1984; Wodinsky, 2008). This disparity might be especially prominent among the octopods (Cephalopoda: Octopoda). For example, at any given time males of the giant Pacific octopus, *Enteroctopus dofleini*, (Hochberg, 1998) carry up to approximately ten spermatophores (Mann et al., 1970), each of which can take over an hour to be placed during mating (Anderson et al., 2003). By contrast, females of this species spawn up to 100,000 eggs, and are not limited in the number of males with which they can mate (Hartwick, 1983). The constraint of having a male reproductive capacity that could potentially be limiting might lead to a high investment by male cephalopods towards their gametes, and therefore a system in which male investment towards mate selection might influence reproductive success of individuals within a species. Accordingly, pre-copulatory mate choice by males has been observed in the algae octopus, *Abdopus aculeatus*, (d'Orbigny, 1834) where males preferentially guard and have longer bouts of male-male aggression over larger females that are likely to have higher egg-laying capacities (Huffard et al., 2008; 2010). Simlarly, in the California two-spot octopus, *Octopus bimaculoides,* (Pickford & McConnaughey, 1949) there is increased male-male aggression over immature females, which are likely to hold fewer sperm from competing males (Mohanty et al., 2014).

Female octopods store sperm internally in the oviducal glands until they are ready for egg deposition, which is the time when fertilisation occurs (Mangold, 1987; Hanlon & Messenger, 1998). This system can lead to muliple paternity (Morse, 2008; Voight & Feldheim, 2009; Quinteiro et al., 2011). Sperm competition, in the forms of sperm removal, sperm-loading and mate guarding have been documented amongst male cephalopods (Hanlon et al., 1999; Iwata et al., 2005; Wada et al., 2005b; Wada et al., 2006; Huffard et al., 2008). However, studies investigating the differential time investment that males allocate towards copulating with different females, based on either female novelty or recent mating history of females, are limited (c.f. Cigliano, 1995; Wada et al., 2010). Males of both an unidentified pygmy octopod and the kisslip cuttlefish, *Sepia lycidas*, (Gray, 1849) have been observed to spend longer copulations, either performing sperm removal, or transferring more spermatophores with females that had recently mated with a competing male (Cigliano, 1995; Wada et al., 2010).

As the availability of spermatophores are limited for most male cephalopods (Mann, 1984; Wodinsky, 2008), it is predicted that the strategic allocation of spermatophores and/or time by males might be commonplace amongst the Cephalopoda. Relevant models of sperm competition, where sperm supply is limited, imply that male cephalopods could potentially achieve optimal fertilisation success by investing less time copulating with females that they have already mated with (Parker, 1970), with females that are holding less sperm from competing males and therefore pose less risk of sperm competition (Ball & Parker, 2007), and/or when additional factors such as male mating order might give males an inherent advantage towards successful fertilisation (Parker, 1990). Likewise, male cephalopods should be expected to invest more time and/or spermatophores with novel females and females posing a high-risk of sperm competition (Parker, 1970; 1990; Parker et al., 1997; Ball & Parker, 2007).

The southern blue-ringed octopus (*Hapalochlaena maculosa*) presents a model for addressing hypotheses concerning cephalopod mate choice for several reasons: 1) Copulations in this species are protracted compared to copulation times reported across other cephalopod taxa (see review in Hanlon & Messenger, 1998), suggesting that sperm-loading, sperm removal or male monopolization of females might be important in this species; 2) Copulations can either be terminated by the male or female, suggesting that either sex can regulate their time and/or potential gamete investment during copulation; and 3) Sexually mature, virgin males bear approximately fifty spermatophores at any given time, and sexually mature females have approximately the same number of eggs (Tranter & Augustine, 1973), suggesting that male strategic allocation of their spermatophores might be critical to male

reproductive success. Additionally, this species is small and easy to maintain in captivity, and adults are often found in very close proximity of each other in the wild (P. Morse, unpubl. data) making it feasible to recreate realistic population densities in laboratory settings. Finally, this species also has synchronous seven month life-cycles with a terminal breeding season (Tranter & Augustine, 1973), making it easy to obtain sufficient numbers of sexually mature adults for simultaneous study.

One of the limitations to studying cephalopod mating systems is that it is difficult to make long-term observations of most species in a natural setting. While field studies have been possible for some large decapods that spawn in aggregations (Corner & Moore, 1981; Hanlon et al., 1997; Hall & Hanlon, 2002; Jantzen & Havenhand, 2003), and a diurnal octopus of moderate size, (Huffard, 2007; Huffard et al., 2008; 2010), small or highly cryptic cephalopods may be more efficiently studied in a semi-natural setting. As with many cephalopod taxa (Boyle, 1987), *H. maculosa* often live in subtidal and usually turbid water, and are nocturnal, making it currently impractical to gain long-term observations of natural mating behaviour for this species in the wild. Therefore, this study aimed to describe key aspects of both male and female mate choice behaviours in *H. maculosa* by reporting on focal animal observations made under laboratory-simulated natural conditions using infrared closed-circuit television (CCTV) and an experimentally manipulated operational sex ratio (OSR). Specifically, this study aimed to address the following questions relevant to the mating behaviour of *H. maculosa* within simulated natural conditions:

Approach Behaviour:
   a) Does either sex make more approaches to conspecifics within trials, and is this affected by the OSR?

Copulatory Success:
   b) Can any measurable morphological or behavioural trait be linked to higher copulatory rates or time spent copulating by either males or females, and is this affected by the OSR?

Copulation Terminations:
   c) Are males more likely to terminate copulations early with females based on the novelty of the female, or her recent mating history in a manner consistent with predictions of sperm competition and/or strategic allocation of finite spermatophores?

Male-Male Mount Comparisons:
   d) Do males attempt to mount other males, and if so how does the frequency, success and duration of male-male mounts compare to male-female mounts?

METHODS
Animal Acquisition and Maintenance
Wild adult *H. maculosa* (males: n = 12; females: n = 12) were sourced from false-shelter traps and from the by-catch of commercial fishermen between the Mandurah and Cockburn Sound coastlines in Western Australia (32°17'59" S, 115°39'4" E ± 40 km) from November 2013 to June 2014. A variety of false-shelter traps were used to obtain animals, and ranged from 20 mm lengths of plastic pipe (19 – 25 mm diameters) and concrete traps adapted from Schafer (2001). The cavity and entrance sizes were modified versions of the concrete traps used in (Schafer, 2001), which corresponds with the size of shells and structures that *H. maculosa* are observed to inhabit in the wild (P. Morse personal observations). Two sizes of concrete trap were used in this study to limit the size bias in collections. Small concrete traps had 50 x 30 mm cavities with 10 x 20 mm entrance holes. Large concrete traps had cavities

sized 70 x 45 mm with 15 x 25 mm entrances. Animals were successfully found using all trap types, and *H. maculosa* also readily used the same trap types as shelters within the lab. Animals sourced from by-catch were obtained through commercial fishermen that fished for the gloomy octopus, *Octopus tetricus*, (Gould, 1852) under the license of the Fremantle Octopus Company. Commercial fishermen used a combination of larger false-shelter traps (approximately 20 cm in diameter), and Trigger Traps designed by Octopus Technologies PTY LTD. All animals were taken from between 3 – 28 m of water depth.

Animals were sourced under Western Australia DPaW permit: SF00963. The use and treatment of the animals were approved by the James Cook University Animal Ethics Committee (Approval Number: A1850). All animals were housed within individual 1 L plastic containers connected to a closed flow-through system with a 1,000L sump at Fremantle Octopus Company facilities in O'Conner, Western Australia (WA). Seawater was obtained from Cockburn Sound, WA where most of the animals were sourced, and water parameters we continuously maintained at 22 ℃ and between 34 – 35 ppt salinity. Male and female containers were separated by an opaque divider, and activated carbon was used to neutralise odours in seawater before entering animals' individual containers to limit animals' awareness of any pre-existing OSR prior to trials (see Kvarnemo & Ahnesjo, 1996). Each animal was given an appropriately sized shell for use as a den, and animals were fed *ad libitum* with sections of prawn and occasional live crabs. ReefOne™ biOrb fluorescent LED lights were used to simulate daylight for 14 hours per day, which corresponded to local daylight hours when trials began. All individuals were of adult size on capture and so were likely to have mated in the wild prior to experiments. Therefore all animals were maintained under these laboratory conditions for a minimum of two weeks prior to trials to help minimise any bias of different mating histories prior to capture.

Focal animal observations during copulatory behaviour trials were made in a larger experimental tank that was set up to simulate the substrate as similarly as possible to where *H. maculosa* were sourced. The bottom of the tank was 1 m² and had a water depth of 50 cm. The bottom of the tank was lined with sandy rubble. Twelve shells of various shapes and sizes, all large enough for *H. maculosa* to hide in, were scattered haphazardly across the tank floor. An aerator was used to keep water oxygenated during focal animal trials. Animals were fed *ad libitum* with sections of prawn throughout trials, and excess waste was removed from the experimental tank daily using a net.

Animal Measurements

Morphological traits were measured on all animals one day prior to entering trials. Wet weights were recorded using a digital scale. Mantle length (ML) and interocular width were recorded to the nearest mm using gloves and a ruler, while keeping the animal out of the water for a maximum of two mins. Male ligula lengths (Robson, 1929) were very small and had little variability (Mean = 2.08 mm ± 0.23 S.E.), so were not included within analyses. Length measurements were confirmed by additionally photographing the animal over a grid of 1 cm squares. Individual colouration patterns, markings and arm injuries were noted to aid in identifying individuals during trials (Adamo et al., 2000; Huffard et al., 2008).

Copulatory Behaviour Trials

Copulatory behaviour trials consisted of focal animal observations recorded for six animals at a time within the experimental tank. In total, 24 animals were used to make up four distinct trials, each having one of three levels of OSR. A male-biased OSR trial was comprised of four males and two females; a female-biased OSR trial contained two males and four females; and two equal OSR trials both contained three animals of each sex. Each of the 24 animals was randomly selected from the available animals housed in the laboratory at the time of the trial, and each animal only entered one trial. All animals had a mantle length of at

least 20 mm as this was the minimum size of animal observed to mate during pilot studies. Male sizes ranged from 2 – 9 g wet weight, (26 – 38 mm ML; 7 – 14 mm IO). Female sizes ranged from 1 – 12 g wet weight (20 – 42 mm ML; 5 – 17 mm IO). The six animals entering each trial were put into plastic containers with holes in them that were suspended within the experimental tank for 12 hours preceding the start of each trial in order to acclimate them to the new tank.

Each trial was planned to run for five days. However, in the first equal OSR trial one of the males became almost completely inactive on the fourth day after copulating for 197 minutes with a female much larger than himself. Therefore this trial was terminated after 3.28 days, and a new equal OSR trial was created using six new animals, and was allowed to run for another two days giving a total of 5.28 days of data for equal OSR trials. An overhead CCTV camera (Anran: High Resolution SONY CCD 700TVL Waterproof 78IR Zoom: 2.8 – 12 mm) was used to record all trials continuously to an external hard drive. Because this species is nocturnal (P. Morse personal observations) infrared video was used to monitor interactions taking place during the ten night-time hours each day. Visual checks were made daily to identify animals based on their individual markings recorded during animal measurements, and their locations within the tank in order to ensure that IDs were correct during video playback.

Focal Animal Video Observations

During video playback, behaviours were scored for each of the 24 individuals among the total 15.28 days of focal animal observations in the four trials. The following behaviours were scored: number of approaches made by each animal; first animal to make contact after an approach; retreating individual after any interaction; male mount attempts with females; female receptivity to male mount attempts; successful male-female copulations; copulation durations; individuals terminating each copulation; and the identification of individuals in all interactions. Definitions of female receptivity and female-terminated copulations are outlined for the greater blue-ringed octopus, *Hapalochlaena lunulata*, (Quoy & Gaimard, 1832) by Cheng and Caldwell (2000), and were used for categorising the behaviours within this study. In short, females were considered receptive to male copulation attempts if there was no grappling phase or obvious attempt to retreat between male contact and a successful male mount. It was not possible always possible to observe hectocotylus insertion due to the small size of the animals and the fixed camera angle. Therefore, male-female copulations and male-male mounts were considered successful if the mount lasted for a minimum of 30 s. Similarly, it was not possible to enumerate spermatophore release, and so this was not addressed within analyses. Copulations were considered terminated by the female when the female was observed using her arms to push the male off of her. Male-terminated copulations were categorised by the male passively unmounting the female without female instigation. It was not possible to identify which sex terminated two of the copulations during video playback, and these copulations were omitted from analyses that used copulation termination as a factor.

Behavioural Analyses

A total of 29 male-female copulations and 557 approach/contact/retreat interactions were observed among the four trials. Where appropriate, measures were scaled to daily rates to accommodate the different lengths of observation time for animals in separate trials. Copulation durations were transformed to a normal distribution using a log-scale transformation prior to analyses. Some animals did not copulate during trials, and so time spent copulating per day for individual animals was transformed to a log + 1 scale to normalise this distribution that contained values of zero. Frequencies of copulations

terminated by either sex were compared between each level of OSR using Fisher's Exact Test, and all other behavioural comparisons were made using general linear modelling (GLM).

Approach frequencies between each pair of animals within trials were compared using Negative Binomial Models with response values offset by the log-value of days that animals were observed for, as this test is robust against data that has a high residual deviance (Jones et al., 2013). Copulatory rates and male-female copulation attempts, being frequency data, were analysed using GLMs fitted to a Poisson distribution. Proportional data were analysed using Logistic Regression. Comparisons made using daily time spent copulating per animal as the response variable were analysed using a Fixed-Effects ANOVA as each animal only had one data point within these analyses. Most animals copulated more than once, so all comparisons of copulation durations among the 29 observed copulations were analysed using a Mixed-Effects Model with the identification of individual males and females both set as random effects to account for individual variation between animals. Comparisons of male-male mount attempts to male-female mount attempts within different levels of OSR were also performed using a Mixed-Effects Model with male ID as a random effect to account for males that made attempts to mount both males and females. Additionally, as in this analysis male-male mount attempts were greatly influenced by trial OSR, individual rates of daily mount attempts towards males and females were divided by the relevant number of other males and females in the trial respectively to make the rates comparable between trials containing different OSRs. Finally, these OSR-corrected daily rates were fitted to a 'square root + 1' transformation to normalise the distribution of these frequency data containing zeros.

Although all animals had a minimum mantle length of 20 mm, two of the females that had wet weights of less than five grams were unreceptive to all male copulation attempts and did not copulate during trials. One of these females was from the female-biased OSR trial and the other was from the second equal OSR trial. These two females might have been sexually immature and so their rejections of attempts by males to copulate were omitted from comparisons of female receptivity to male size. All males gained copulations within trials, and males made copulation attempts with all females. All statistical analyses were carried out using S+ software under license to James Cook University.

RESULTS
Approach Behaviour

With very few exceptions, focal animals spent all daylight hours hiding within shells or under gravel. Daytime behaviours consisted only of occasionally changing shelter locations between shells or gravel, or approach/copulatory behaviours during the first two hours after animals were placed in a trial. All other approach, feeding and copulatory behaviours occurred during simulated night-time hours when interactions were recorded using infrared CCTV. All animals were relatively active during night-time hours. Males made significantly more approaches to conspecifics within trials than females (Negative Binomial Model: $X^2 = 11.284_{117}$, p < 0.001; Figure 1). Male approach frequencies were significantly affected by the OSR of the trial, in that individual males made more approaches to conspecifics in trials that contained fewer females (Negative Binomial Model: $X^2 = 4.159_{58}$, p = 0.041; Figure 1). However, female approach frequencies were unaffected by OSR (Negative Binomial Model: $X^2 = 0.218_{58}$, p = 0.64; Figure 1). Individual approach fequencies were independent of animal size among both males (Negative Binomial Model: $X^2 = 1.245_{57}$, p = 0.265) and females (Negative Binomial Model: $X^2 = 0.025_{57}$, p = 0.874).

Among pairwise approach combinations between all individuals in trials, the sex of the approached animal had no effect on approach frequencies among either male (Negative Binomial Model: $X^2 = 0.423_{58}$, p = 0.515) or female (Negative Binomial Model: $X^2 = 0.333_{58}$, p = 0.564) approaches. This suggests that animals within trials were either unable to

discriminate the sex of consepecifics while approaching, or chose to approach both sexes equally.
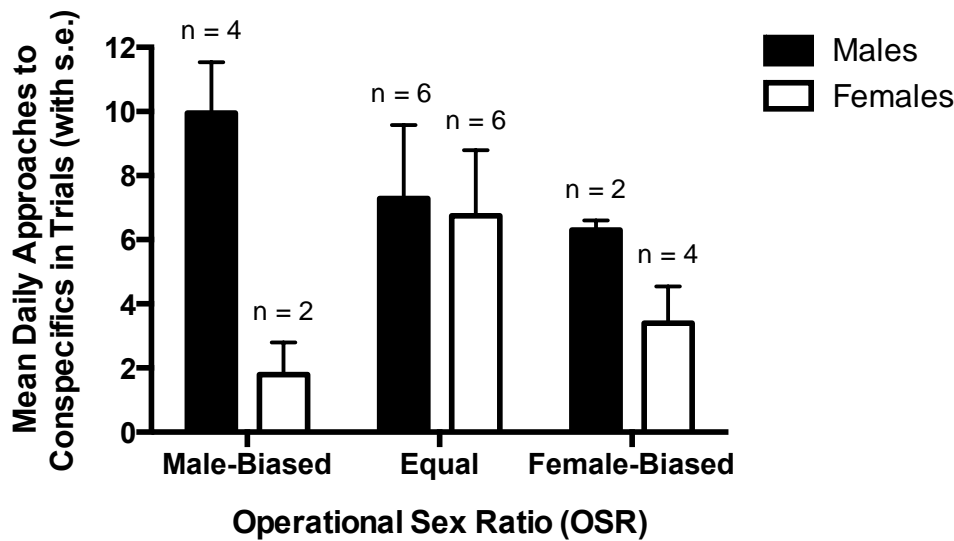

Figure 1. Mean male and female approach rates (approaches per day per individual) by OSR treatment. Males made significantly more approaches towards conspecifics within trials than females, and male approaches were significanty affected by the OSR of the trial.
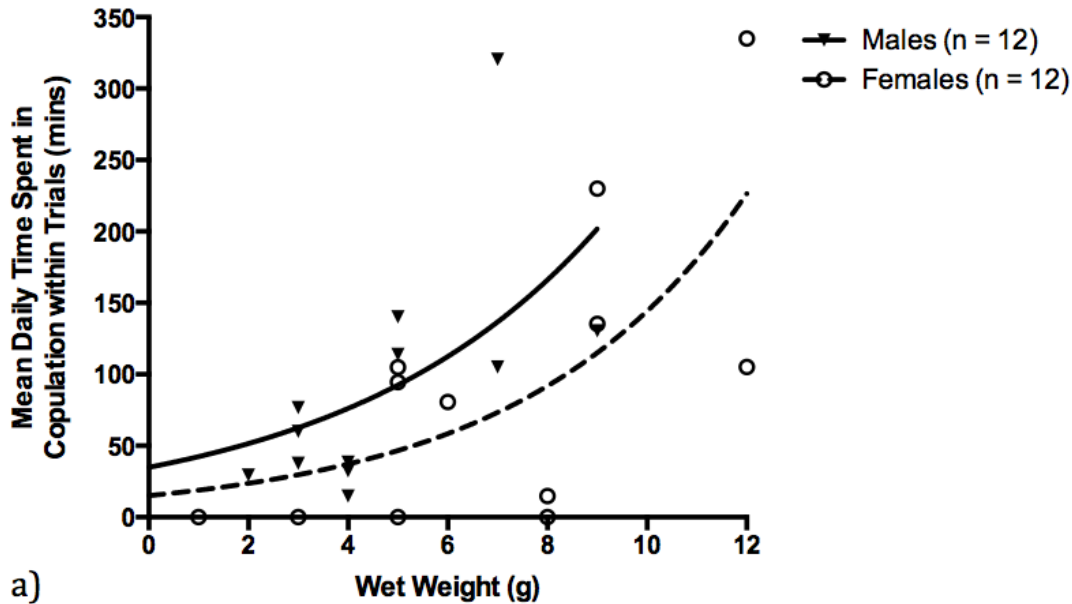
Copulatory Rates

A total of 29 successful male-female copulations were observed among the four trials. Observations were consistent with behaviour recorded by Tranter and Augustine (1973), in that males mounted females by wrapping their arms around their mantle and inserting the hectocotylus through the female's aperture. Although males made more approaches overall (Figure 1), and female rejection of male copulation attempts was common (see below), successful copulations among all trials were initiated equally by approaches from both sexes (Generalised Linear Poisson Model: $X^2 = 0.311_{10}$, p = 0.577). Copulation durations ranged from 38 to 348 mins (Mean = 117.66 mins ± 14 S.E.). All males copulated during trials, however females only participated in copulations at a minimum of five grams wet weight (Figure 2a).
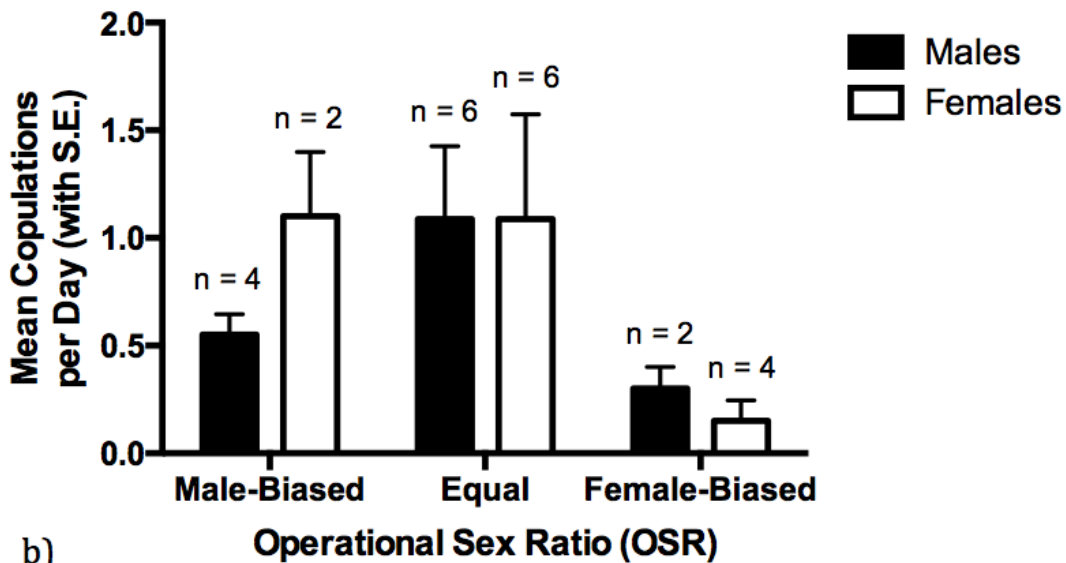
Copulatory rates (mean per 24 h) within trials increased with body mass for both males (Generalised Linear Poisson Model: $X^2 = 5.216_9$, p = 0.005) and females (Generalised Linear Poisson Model: $X^2 = 12.791_9$, p < 0.001). Similarly, mean daily time spent in copulation increased with size for both males (Fixed-Effect ANOVA with a Log + 1 Transformation: F = $9.121_{1\ 8}$, p = 0.017; Figure 2a), and females (Fixed-Effect ANOVA with a Log + 1 Transformation: F = 9.645_{18}, p = 0.015; Figure 2a). Additional measures of size, mantle length (Fixed-Effect ANOVA with a Log + 1 Transformation: F = $8.458_{1\ 10}$, p = 0.016) and interocular width (Fixed-Effect ANOVA with a Log + 1 Transformation: F = $8.116_{1\ 10}$, p = 0.017) had significantly positive relationships with time spent copulating by females. However, these morphological traits were highly correlated with body mass among females (Mantle Length/Wet Weight: r = 0.908; Interocular Width/Wet Weight: r = 0.886). Therefore wet weight was used to represent body size in following analyses. These traits measured on males were less correlated to body mass (Mantle Length/Wet Weight: r = 0.254; Interocular Width/Wet Weight: r = 0.847) and did not have significant relationships with average time spent copulating by males (Mantle Length: Fixed-Effect ANOVA with a Log + 1 Transformation: F = $271_{1\ 10}$, p = 0.254; Interocular Width: Fixed-Effect ANOVA with a Log + 1 Transformation: F = $3.825_{1\ 10}$, p = 0.079).

Trial OSR had no significant effect on average time spent copulating per day among either males (Male-Biased OSR: Mean = 46.4 min ± 23.2 S.E.; Equal OSR: Mean = 132.48 mins ± 54.08 S.E.; Female-Biased OSR: Mean = 60 mins ± 42.43 S.E.; Fixed-Effect ANOVA with a Log +

1 Transformation: $F = 0.023_{18}$, $p = 0.884$), or females (Male-Biased OSR: Mean = 85.7 mins ± 60.6 S.E.; Equal OSR: Mean = 132.48 mins ± 54.08 S.E.; Female-Biased OSR: Mean = 30 mins ± 15 S.E.; Fixed-Effect ANOVA with a Log + 1 Transformation: $F = 3.754_{18}$, $p = 0.089$). However average female copulatory rates in trials were significantly affected by OSR (Generalised Linear Poisson Model: $X^2 = 13.79_{10}$, $p < 0.001$), with females gaining more copulations in trials that contained more males (Figure 2b). Contrastingly, male copulatory rates were not significantly affected by OSR (Generalised Linear Poisson Model: $X^2 = 0.692_{10}$, $p = 0.406$; Figure 2b).

Figure 2. a) Time spent copulating per day increased with size for both males and females. Copulation times were unaffected by trial OSR. The solid and broken lines represent linear regressions to log + 1 transformed data: $y = e^{((0.274x + 2.932)-1)}$ for males ($p = 0.017$); and $y = e^{((0.449x - 0.007)-1)}$ for females ($p = 0.028$) respectively; b) Male copulatory rates were not significantly affected by trial OSR, however females had significantly more copulations within trials containing more males.

Copulation Terminations

Among the 29 observed copulations, two copulations were terminated either inside or behind a shell and so the terminating member could be identified for a total of 27 copulations during trials. Among these observations, 15 copulations were terminated by females and 12 by males. Copulations were terminated equally by both sexes and there was no effect of OSR on these frequencies (Fisher's Exact Test: d.f. = 2, $p = 0.699$). Among focal animals, there was

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no effect of female wet weight on the likelihood of a female to terminate copulations (Logistic Regression: $X^2 = 0.874_5$, p = 0.35). Larger males did terminate significantly more copulations than smaller males (Logistic Regression: $X^2 = 4.905_9$, p = 0.027). However, this pattern was driven by larger males having more copulations than smaller males (Figure 2), and males being more likely to terminate copulations during subsequent matings (see Male Mating Behaviour below). Among observed copulations, there was no effect of size difference between males and females influencing the sex that terminated copulation (Logistic Regression with a Binary Response: $X^2 = 0.834_{25}$, p = 0.361). After accounting for variability among individuals as a random effect, copulations terminated by males were significantly shorter than copulations terminated by females (Linear Mixed-Effects Model with male and female identifications as random effects and a log transformation of Copulation Time: F = $6.301_{1\,6}$, p = 0.046; Figure 3).
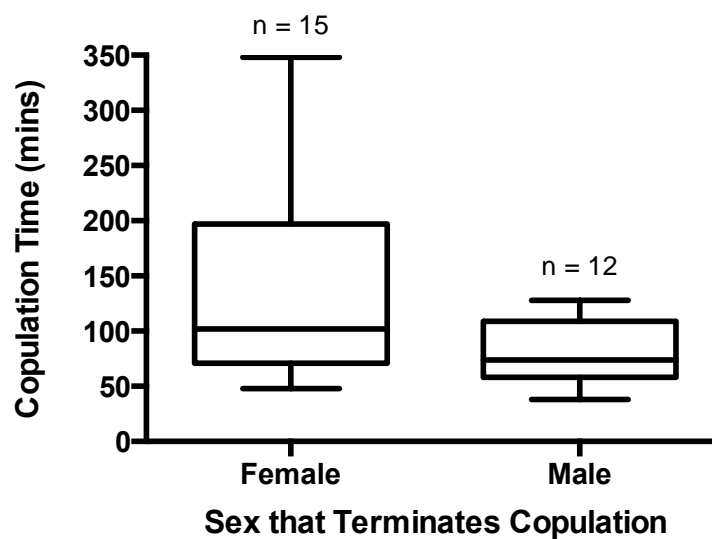

Figure 3. Male terminated copulations were significantly shorter than female terminated copulations.

Female Mating Behaviour

Female copulation times decreased significantly during subsequent copulations within trials (Linear Mixed-Effects Model with male and female IDs as random factors and a log transformation of Copulation Time: F = $9.519_{1\,10}$, p = 0.012). Females were often unreceptive to male mounting attempts by pulling their arms over their mantle apertures, grappling with the male and/or attempting to retreat when the male tried to mount. This mode of mate rejection occurred on 25 occasions among trials, and on nine of these occasions the male managed to mount and copulate with the female anyway. Female receptivity to males was similar between all levels of OSR (Logistic Regression: $X^2 = 0.903_{10}$, p = 0.342). Excluding copulation attempts with females smaller than five grams, male size had no effect on female receptivity (Logistic Regression: $X^2 = 2.655_9$, p = 0.103). Female receptivity to copulations did significantly increase with female size (Logistic Regression: $X^2 = 9.155_9$, p = 0.002; Figure 4a). Additionally, there was some evidence for females to mate for longer with larger males (Figure 4b). After accounting for individual variation as a random effect, there was a significant interaction between the sex that terminated copulation and male size impacting on copulation time (Linear Mixed-Effects Model with male and female IDs as random effects and a log transformation of Copulation Time: F = $9.712_{1\,7}$, p = 0.017).
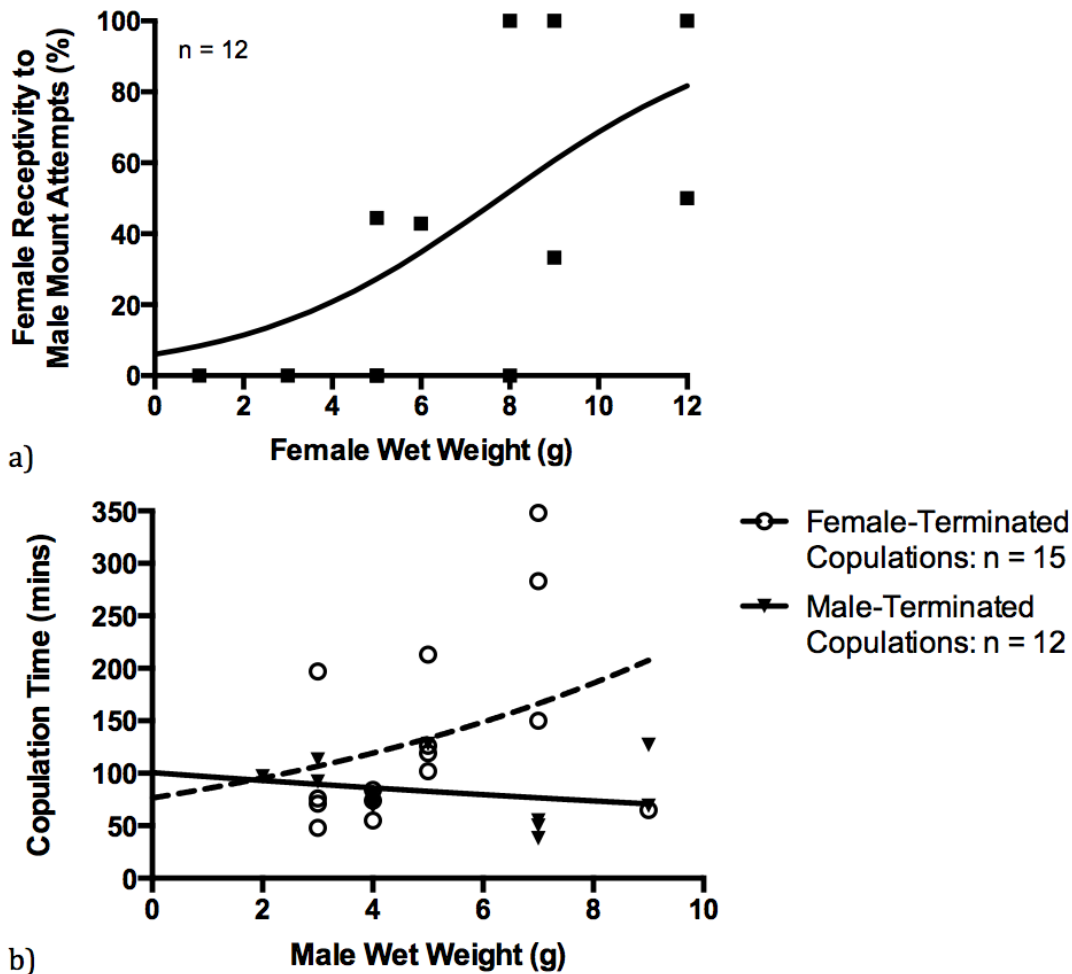
Figure 4. a) Larger females were receptive to male copulation attempts significantly more often than smaller females Females did not begin being receptive to copulations until they reached a minimum of five grams wet weight. The solid line represents the logistic regression: $y = 1 / (1+e^{-(3.354x - 2.75)})$; p = 0.002; b) There was a significant interaction between the sex that terminates copulation and male size impacting on copulation time. Among female-terminated copulations, copulations tended to be longer with larger males, while the lengths of male-terminated copulations were independent of male size. The solid and broken lines represent Linear Mixed-Effect Models fitted to log transformed data: $y = e^{(-0.096x + 4.907)}$ for male-terminated copulations (p = 0.017); and $y = e^{(0.172x + 3.889)}$ for female-terminated copulations (p = 0.047) respectively.

Male Mating Behaviour

Male-male physical aggression over females and mate guarding were not observed during this study. Instead all male-male interactions were confined to contact-retreat behaviours or mount attempts. Male-female copulation times decreased significantly with all subsequent copulations during trials (Linear Mixed-Effects Model with male and female identifications as random factors and a log transformation of Copulation Time: $F = 8.084_{1\,10}$, p = 0.018). Larger males attempted to copulate with females more frequently than smaller males within trials (Generalised Linear Poisson Model: $X^2 = 7.463_9$, p = 0.006; Figure 5a). However, female size had no effect on the number of copulation attempts she received (Generalised Linear Poisson Model: $X^2 = 1.357_9$, p = 0.244).

Among the 27 observed terminations of copulation, males terminated copulations based on different criteria depending on if it was his first or subsequent copulation within a trial. During first copulations of all males in a trial, they always waited for the female to terminate the copulation if the female had not yet copulated with another male during the trial. In contrast, males always terminated their first copulations early if they were mating with a female that had mated with a different male previously in the same trial (Logistic

Regression: $X^2 = 13.863_8$, p < 0.001; Figure 5b). Among all subsequent male copulations, female novelty significantly influenced the likelihood of a male to terminate the copulation early (Logistic Regression: $X^2 = 8.614_{15}$, p = 0.003; Figure 5c). Males were significantly more likely to terminate copulations with females that they had already mated with during the trial, and copulation times between repeating pairs of males and females were significantly shorter than copulations between novel pairs (Linear Mixed-Effects Model with male and female IDs as random effects and a log transformation of Copulation Time: $F = 7.079_{1\,10}$, p = 0.024; Figure 5d). Among the eleven copulations between repeating pairs of males and females, males were significantly more likely to terminate the copulation if they were the last male to have mated with the female but were more likely to wait for the female to terminate if the female had last mated with a different male (Logistic Regression: $X^2 = 4.18_9$, p = 0.041). Additionally, the lengths of male-terminated copulations varied significantly according to recent female mating history. Male-terminated copulations were significantly longer with females that had previously mated with more competing males during the same trial (Linear Mixed-Effects Model with male and female IDs as random effects and a log transformation of Copulation Time: $F = 9.334_{1\,6}$, p = 0.022).
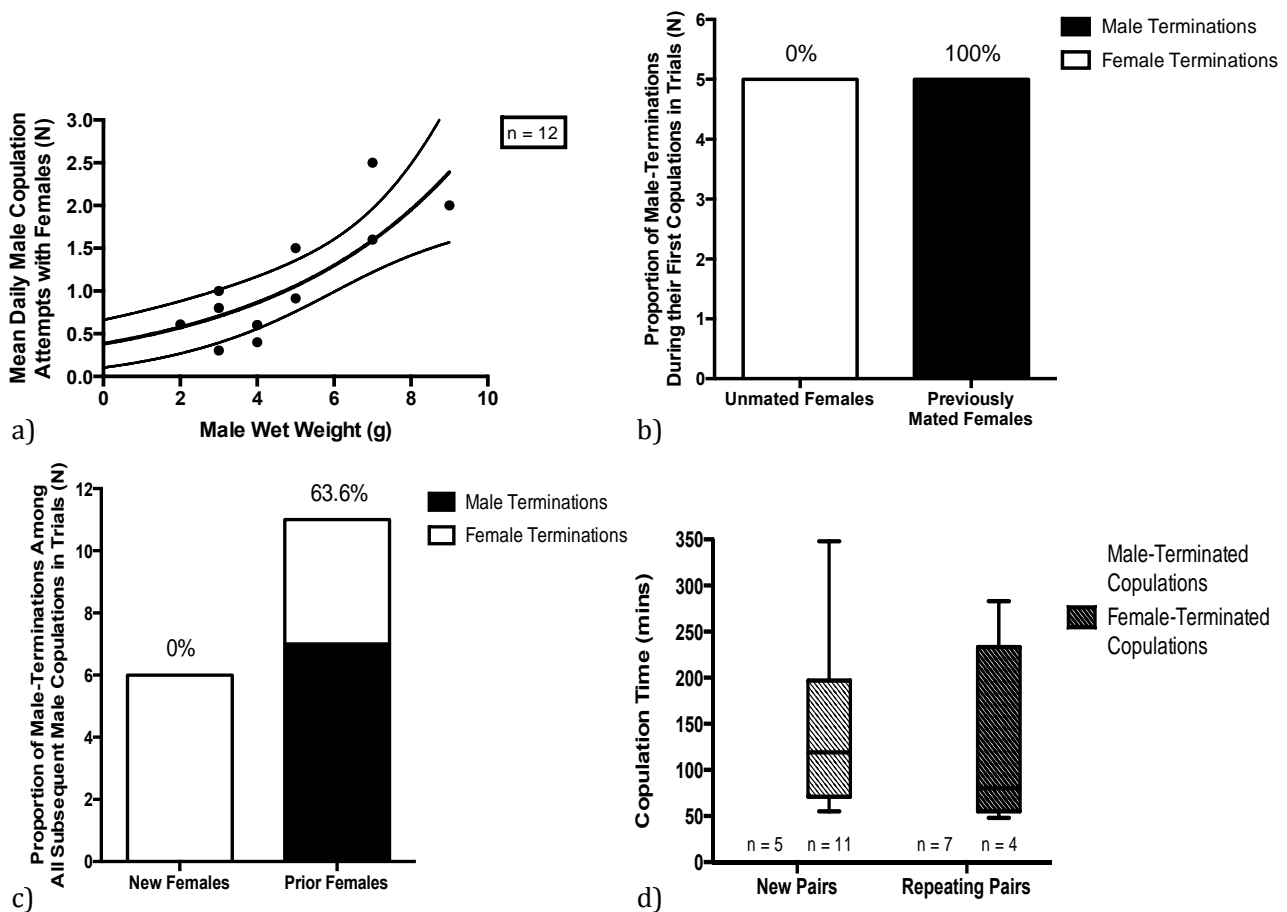

Figure 5. a) All males attempted to mate with females, and among all trials larger males attempted to copulate with females more frequently than smaller males. The solid line represents the Poisson regression: $y = e^{(0.318x - 1.088)}$; p = 0.006. The broken lines represent the 95% confidence limits of the equation; b) Among male's first copulations of the trial, the male was always the terminating member when the female had previously mated with another male in the trial. When it was the first copulation of the trial for both the male and the female, the male always waited for the female to terminate the copulation; c) Among all male subsequent copulations, males were significantly more likely to terminate the copulation if they had previously mated with the female, and would always wait for the female to terminate the copulation if he had not yet mated with her; d) After accounting for individual variation among individuals as a random effect, copulation times during subsequent copulations between males and females that had already previously mated together were significantly shorter than copulations between new pairs of males and females. Sample sizes were too small to detect differences in copulation times within only male or female-terminated copulations.

Male-Male Mount Comparisons

Male-male mounts were frequent among trials. After making daily rates of male attempts to mount comparable between trials by dividing daily rates of male-male and male-female mount attempts by the number of other males and females in trials respectively, the overall frequencies of male-male and male-female mount attempts were similar (Linear Mixed-Effects Model with male ID as a random effect and a 'square root +1' transformation of OSR adjusted daily mount attempts: $F = 2.89_{1,10}$, $p = 0.12$). However, there was a significant interaction between trial OSR and the sex that males most frequently tried to mount (Linear Mixed-Effects Model with male ID as a random effect and a 'square root +1' transformation of OSR adjusted daily mount attempts: $F = 8.099_{1,10}$, $p = 0.017$). This interaction was driven by there being more than twice as many male-male mount attempts than expected within the male-biased OSR trial (Figure 6). In contrast to male-female mounts, males were never receptive to being mounted by another male, and this was usually followed by grappling and/or retreat behaviour from one of the males. This led to the success rate for male mount attempts to be significantly less for male-male mounts than male-female mounts (Logistic Regression: $X^2 = 26.057_{18}$, $p < 0.001$; Figure 7a). Although successful male-male mount durations ranged from 2 to 162 mins, average male-male mount times were significantly shorter than male-female mounts (Linear Mixed-Effects Model with male and female IDs as random effects and a log transformation of Mount Time: $F = 48.258_{1,17}$, $p < 0.001$; Figure 7b).
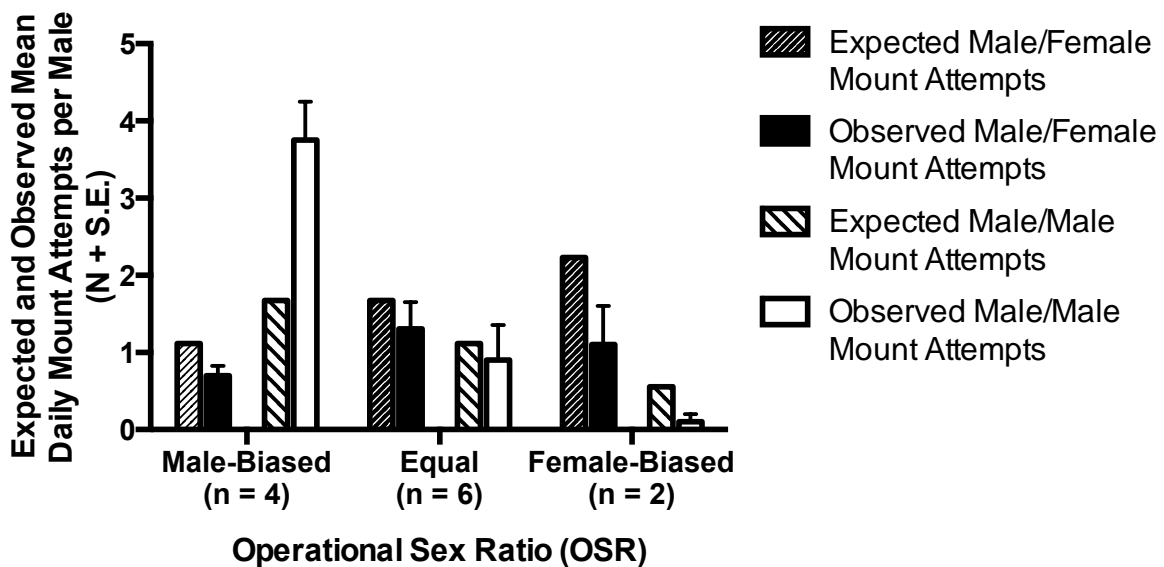

Figure 6. Male-female and male-male mount attempt rates were similar among all trials. However there were more than twice as many male-male mount attempts than expected in the male-biased OSR trial. Expected attempt rates are shown for illustrative purposes only and were calculated using the average daily total mount attempts per male and multiplying by the ratio of other males and females in trials to predict expected male-male and male-female mount rates respectively. In the male-biased OSR trial, expected daily rates = 2.787*(2/5) for male-female mounts and 2.787*(3/5) for male-male mounts; Equal OSR Trial expected daily rates = 2.787*(3/5) for male-female mounts and 2.787*(2/5) for male-male mounts; and in the female-biased OSR trial, expected daily rates = 2.787*(4/5) for male-female mounts and 2.787*(1/5) for male-male mounts.
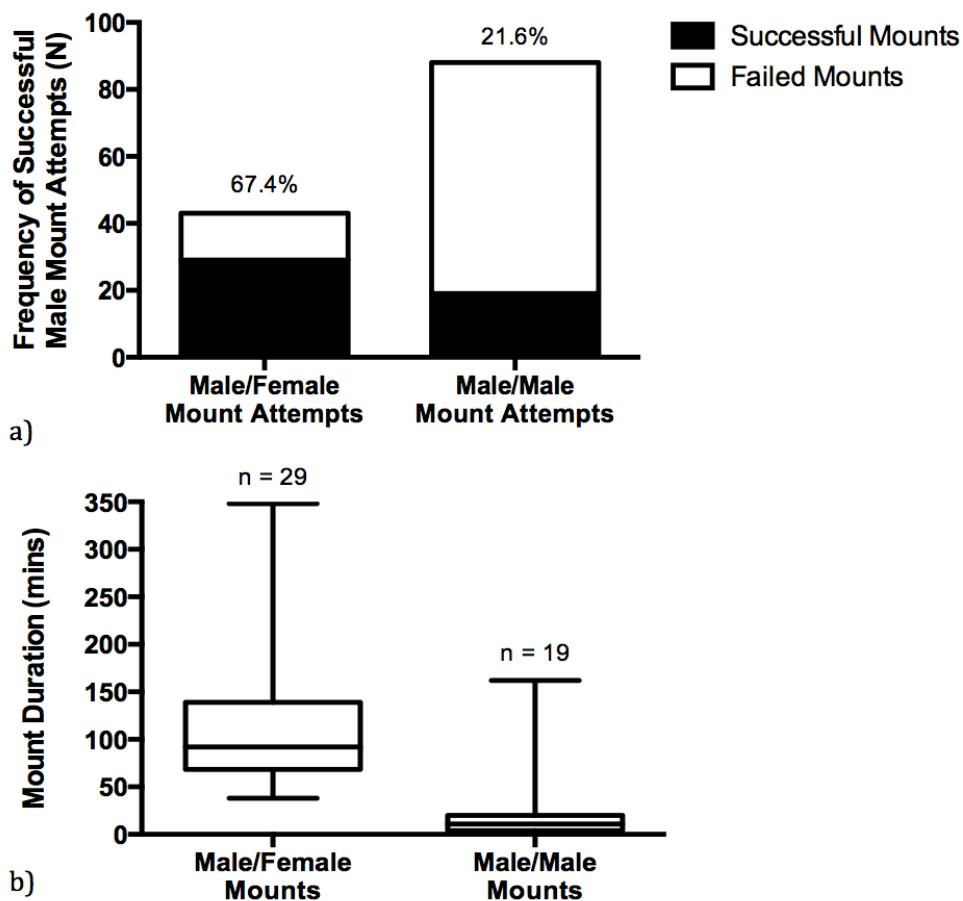
Figure 7. a) Male mount attempts were significantly more likely to be successful with females than with other males; b) Successful male-male mount durations were significantly shorter than successful male-female mounts.

DISCUSSION

Laboratory observations of focal animals in this study indicated that, when sexually mature, both male and female *H. maculosa* approach and contact nearby conspecifics, which often leads to an attempt to mount by males, and copulation. Males made more approaches than females, particularly when the availability of females was low, suggesting that males expend more effort than females to initiate copulation. This result is consistent with patterns observed across most animal groups where anisogamy exists (Kodric-Brown & Brown, 1987). Both males and females were approached in similar frequencies, and male-male mount attempts were very common, suggesting that *H. maculosa* could not discriminate the sex of approached conspecifics within trials. This observation supports findings by Cheng and Caldwell (2000), where male *H. lunulata* was found to attempt mounting other males as readily as female conspecifics.

The apparent lack of sex recognition in *Hapalochlaena* spp. contrasts with field observations of *A. aculeatus*, which recognised the sex of conspecifics from a distance (Huffard et al., 2008), and laboratory experiments with *O. bimaculoides*, which discriminate conspecific sex based on odour cues (Walderon et al., 2011). Yet even in *A. aculeatus*, male-male mating attempts did occur in low frequencies and in all cases led to physical aggression (Huffard et al., 2008). The potential use of odour cues were not addressed in this study. However, based on the frequency of male-male approaches and mount attempts, it seems unlikely that male *H. maculosa* use odour cues to identify females to mate with. It is possible that *Hapalochlaena* spp., which have an even shorter breeding window than most other *Octopus* taxa (Tranter & Augustine, 1973; Overath & Boletzky, 1974), have not developed the ability to discriminate the sex of conspecifics. This might be due to the risk of missing an opportunity to mate potentially outweighing the cost of intra-sexual aggression. No male-male aggression has been

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reported within *H. lunulata* (Cheng & Caldwell, 2000), and the only aggression resulting from same-sex mounts in the present study was confined to brief grappling behaviour and never led to noticeable male injury.

Sample sizes within each trial were very low, and may have affected this study's ability to detect differences in mating behaviour between different levels of OSR. For example, there was a consistent trend for females to have longer copulation times in trials containing fewer males. It is possible that a greater sample size might have been able to identify this as a significant pattern. Within the limitations of this study, only three significant behavioural changes were evident between trials containing different OSR: 1) males made more approaches to other individuals when fewer females were available, 2) females had fewer copulations when fewer males were present, and 3) male-male mount attempts were more common than expected in the male-biased OSR trial. The lower female copulatory rates in the female-biased OSR trial coincide with the finding that males approach conspecifics more often in this species, and therefore copulation opportunities for females were reduced in this trial. Similarly, this pattern led to the increase in observed male-male mount attempts during the male-biased OSR trial. Male-biased OSRs have been reported to influence the frequencies of both male sexual displays and male-male competition within other mating systems (Kvarnemo & Ahnesjo, 1996; Jirotkul, 1999; Huffard, 2005). Therefore, it is suggested that the limitation of available females in the male-biased OSR within the present study led to heightened male activity, resulting from increased male motivation to copulate. Consequently, male-male interactions were more common than by chance in the male-biased OSR trial, and this resulted in a greater number of male-male mount attempts per individual than expected.

Body mass was observed to be the strongest factor influencing the difference in copulatory rates among animals in trials. Larger males invested a greater effort than smaller males towards initiating copulation with females by making more copulation attempts, and larger females were more likely than small females to be receptive to these attempts. There was no evidence for precopulatory female preference to mate with larger males. Female receptivity to male copulation attempts was affected by her own size and not by that of the male. This observation is consistent with studies of both decapods and octopods, where females were not observed to discriminate amongst males based on their size (Corner & Moore, 1981; Adamo et al., 2000; Hall & Hanlon, 2002; Huffard et al., 2008; 2010; c.f. Wada et al., 2005a). However, within most of these mating systems larger males still obtained greater copulatory success with females by being more successful in male-male aggressive interactions and in more successfully guarding females.

There was however support for female intra-copulatory preference of males based on size. Among female-terminated copulations there was a tendency for females to mate longer with larger males. Male size may be an indication of sexual maturity for females (Kokko et al., 2003), or it is possible that females might benefit from mating with larger males by having larger offspring with higher fecundity (Kirkpatrick, 1982). Females may be able to bias their offspring paternity towards these males by electing to mate with them for longer, as reported within several insect mating systems (Thornhill & Alcock, 1983). Subsequent studies on paternal size and offspring growth rates and/or gamete counts, as well as assessing paternity patterns amongst genotyped candidate fathers might further elucidate these patterns.

No male preference for females based on size or any other measured physical trait was observed in this study. Males did however adjust their durations of copulation according to both female novelty and recent female mating history. During the males' first copulations in trials they mated for longer and never terminated copulation with a female when she had not yet mated with another male during the trial, thus was less likely to be holding competing sperm. It is not known how many spermatophores were passed during these observations. However, if it is assumed that longer copulation times enable males to transfer more spermatophores to females, as has been reported for some insects (Sakaluk & Eggert, 1996),

then this behaviour was consistent with the risk model outlined by Ball and Parker (2007). These researchers suggested that when sperm supply is limited and female mating status is known, it is advantageous for males to allocate more sperm to virgin females in order to ensure paternity amongst future competing sperm given to that female. Although it is unknown whether any of the females had mated in the wild prior to capture, none of them had copulated for a minimum of two weeks prior to entering trials. Therefore it is noteworthy that males treated these females differently to females that had recently mated during trials. Among subsequent male copulations, a male was only likely to terminate copulation early with a female if he was the last male to have mated with her. Additionally, amongst male-terminated copulations, copulation times significantly increased with the numbers of other males that the female had mated with. These patterns are consistent with predicted behaviours based on models of sperm competition (Parker, 1970; Parker et al., 1997), and with behaviours recorded in both *S. lycidas* (Wada et al., 2010) and an unidentified pygmy octopus (Cigliano, 1995) where males of these species were reported to adjust copulation times, presumably spent performing sperm removal and transferring multiple spermatophores, with females based on whether they were the last male to mate with her.

It is not known whether male *H. maculosa* spent time during copulation removing sperm deposited by previous males, or transferring more sperm of their own to females. However, in a mating system where males have a limiting supply of gametes to use over a limited breeding window, both time and spermatophores are likely to be resources that males allocate strategically (Simmons, 1995; Engqvist & Sauer, 2002; McCartney et al., 2010). The present observations support the possibility that male *H. maculosa* adapt the time spent with a female dynamically based on the likelihood of competing sperm in her oviducts to maximise his chance to still be able to mate with additional females, thereby increasing their overall genetic contribution to the following generation. Future studies are required to examine the mechanisms by which male *H. maculosa* might assess female novelty and mating history. As distance sex recognition was not supported for *H. maculosa* in this study, visual recognition of previous mates also seems unlikely in this species. It is possible that following contact or insertion of the hectocotylus, chemoreceptors as described by Budelmann (1996), might play a role in recognition of the female and/or competing sperm.

It is necessary to acknowledge that the use of two shorter trials for the equal OSR treatment could have affected some of the results. As male copulatory-termination behaviour in particular was heavily dependent on both his own and the female's recent mating history, it is possible that the shorter trials may have missed important patterns that could have been identified if all trials ran for the full five days. However, there were five and ten copulations observed within the two equal OSR trials, which fit within the range of three and twelve copulations observed within the female-biased and male-biased OSR trials respectively. Consequently, males in all trials would have had opportunities to respond to similar conditions of male and female mating history. Additionally, there were no observations of animals behaving outwardly differently with recognised trial members later in trials than when the trials commenced. Therefore it is assumed that any differences in animal behaviour caused by splitting the equal OSR trial into two smaller trials would have been negligible.

Finally, it is also noteworthy that some male-male mounts lasted as long as they did. Same-sex mount durations in the present study were similar to those reported by Cheng and Caldwell (2000) for *H. lunulata*, where the majority of male-male mounts lasted for 5 – 6 mins, but in one case lasted for 44.5 h. These authors reported that spermatophores were not released during same-sex mounts, and this could not be observed during the present study. However, if male *H. maculosa* are apparently selective with their copulation times, and presumably spermatophore investment with females, it remains a mystery why some males engaged in prolonged same-sex mounts to this extent.

CONCLUSION

This study supports the growing literature that the mating systems of octopods are both unique and involve complexities that are yet to be divulged with further observations and experimentation. Specifically, these results support findings by Cigliano (1995) and Wada et al. (2010) in that some male cephalopods appear to strategically regulate their copulation time based on the mating history of the female, presumably to maximise their reproductive output by balancing both their chance of paternity and their ability to successfully copulate with other females. Sperm competition appears prevalent among cephalopod mating systems (Cigliano, 1995; Hanlon et al., 1999; Naud et al., 2004; Shaw & Sauer, 2004; Wada et al., 2005b; Wada et al., 2006; Buresch et al., 2009; Wada et al., 2010; Iwata et al., 2011; Sato et al., 2013). Future studies using molecular markers might identify correlations between paternity and behavioural patterns including copulation duration and chronology. This work will provide the necessary next-steps in understanding the role of sperm competition in the evolution and maintenance of cephalopod mating behaviours.

ACKNKOWLEDGEMENTS

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