The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2015, ••, ••–••. With 2 figures.

Last male sperm precedence in a polygamous squid

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Received 8 January 2015; revised 19 April 2015; accepted for publication 20 April 2015

Differential sperm usage from consecutive matings, or sperm precedence, is vital in determining male reproductive success and the outcome of sperm competition for many organisms. Sperm precedence also has significant consequences for mating system dynamics, including both male and female adaptations for increasing reproductive success and avoiding the costs of mating. Despite sexual selection being a strong driver of reproductive behaviour and morphology in cephalopods, surprisingly few studies have investigated sperm dynamics in this group. To redress this gap, we experimentally quantified sperm precedence patterns in the dumpling squid, *Euprymna tasmanica*, controlling for recent male mating history (first vs. second mating), mating position, and mating frequency. We found that the last male to mate gains an advantage in this system, with the second mating male siring up to 75% of offspring at the beginning of the laying period. The proportion of offspring attributable to the second mating male decreases to 54% by the end of the laying period, potentially as a result of changes in the velocity or number of sperm released from spermatangia over time. There is also significant variation among females in patterns of sperm precedence. This variation was not associated with whether it was the male's first or second mating, male mass, the duration of copulation or the number of pumps (sperm removal behaviour) by the second male. If widespread in cephalopods, last male sperm precedence could help to explain the evolution of mate guarding (or long copulation duration) and sperm removal behaviour in this group. *Biological Journal of the Linnean Society*, 2015, **00**, 000–000.

ADDITIONAL KEYWORDS: cephalopod – competition – euprymna – polyandry – sexual selection – storage.

INTRODUCTION

Post-copulatory sexual selection is an important evolutionary force, as indicated by the numerous morphological and behavioural adaptations of both males and females in relation to sperm competition (Thornhill & Alcock, 1983; Eberhard, 1996; Simmons, 2001a). Sperm competition, the process by which sperm from two or more males compete for the fertilization of a given set of ova, occurs when females mate with multiple males within a single reproductive bout (Parker, 1998). Differential sperm usage from consecutive matings (mating order effect), or sperm precedence, is likely to play an important role in determining male reproductive success and the outcome of sperm competition post copulation (Boorman & Parker, 1976; Lewis & Austad, 1990). Sperm precedence is most commonly measured as P2, the proportion of offspring attributable to the second mating male (Harvey & Parker, 2000) and has been documented in an extraordinary diversity of animal groups (Smith, 1984; Birkhead & Moller, 1992; Simmons & Siva-Jothy, 1993). Patterns of sperm precedence include last male sperm precedence as is common in many insects ($P2 > 0.5$), first male sperm precedence ($P2 < 0.5$) or random sperm mixing ($P2 = 0.5$).

Patterns of sperm precedence may be a function of numerous morphological and behavioural traits, including mate guarding, copulation duration, sperm displacement, manipulative seminal fluids, and cryptic female choice of sperm (Birkhead & Moller, 1992; Simmons & Siva-Jothy, 1993; Birkhead, 1995; Eberhard, 1996; Simmons, 2001a). For example, if the morphology of the female sperm storage organ facilitates sperm stratification and, if the entry and the exit for sperm are the same, a 'last in, first out' last male sperm precedence pattern would be

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expected (Roderick *et al.*, 2003). Male mate guarding may be an adaptive strategy in a system with last male sperm precedence ensuring that the female does not re-mate before reproducing (Roderick *et al.*, 2003). Additionally, if spermatozoa are transferred within packets, temporal dynamics of spermatozoa release may have important implications for sperm precedence patterns. The wide intra- and interspecific variation in P2 values in different species could be a result of the differing effectiveness of these male and female adaptations, and their interaction (Lewis & Austad, 1990; Wilson *et al.*, 1997). Other factors influencing variation in P2 include the mating history of the mating pair and strategic allocation of ejaculates (size and/or number) by males in response to perceived female quality (e.g. mass, mating history, etc.) (Wedell, Gage & Parker, 2002; Wada *et al.*, 2010; Kelly & Jennions, 2011; Wegener *et al.*, 2013). As such, it is important to control for as many of these factors as possible when experimentally assessing variation in P2.

Intraspecific variation in P2 might also provide support for various genetic benefit hypotheses for the evolution of female multiple mating (polyandry) (Yasui, 1998; Simmons, 2001b). This is because sperm competition enables polyandrous females to bias paternity towards males with good genes (Yasui, 1997; Jennions & Petrie, 2000) or away from males with incompatible genotypes (Zeh & Zeh, 1996; Simmons, 2005). Polyandry may also simply increase the genetic diversity of progeny, offering a reproductive advantage under variable environmental conditions (Yasui, 1998; Jennions & Petrie, 2000). Although numerous quantitative experiments that partition variance in P2 among both male and female individuals (and their interaction) are needed to definitively test these hypotheses (Lewis & Austad, 1990; Wilson *et al.*, 1997), assessing patterns of sperm precedence is the first step to understanding sperm dynamics and sperm competition within a female. Temporal assessment of P2 can therefore provide additional insights into these underlying processes (Jones, Adams & Arnold, 2002; Roderick *et al.*, 2003).

Cephalopods are typically highly promiscuous, and females of most species store sperm from multiple males, and for long periods of time (Hanlon & Messenger, 1996; Naud & Havenhand, 2006). However, despite multiple mating being almost ubiquitous in cephalopods, few studies have quantified patterns of sperm precedence in this group. Those that do have focussed exclusively on species with complex mating dynamics, where males use alternative reproductive strategies (such as consorts and sneakers), alternative mating positions, and alternative sperm storage locations (Naud *et al.*, 2004; Iwata, Munehara & Sakurai, 2005; Buresch *et al.*, 2009). All of these factors could lead to different selective pressures on sperm and potentially confound measures of sperm precedence. For example, *Loligo bleekeri* males with different reproductive strategies have corresponding size dimorphic sperm, with small males transferring larger sperm to one location within the female, and large males having smaller sperm that are transfer to another location (Iwata *et al.*, 2011). Although it is important to assess differential success of males with alternative strategies, controlling for factors such as multiple sperm stores and multiple mating positions is critical to provide a clearer picture of underlying sperm precedence patterns among the cephalopods.

We assessed sperm precedence in the dumpling squid, *Euprymna tasmanica*, using a design controlling for recent mating history and frequency. Dumpling squid live in loose aggregations associated with sandy sea beds and seagrass. It is not known how they find or search for mates, nor how often they mate. However, both sexes are polygamous and multiple paternity is common within field laid egg clutches, with a mean $\pm$ SD of 2.73 ± 0.24 (median = 3, range = 2–4) sires per clutch (Squires *et al.*, 2014). Dumpling squid mate year round and no alternative male mating strategies are known. This species has only one observed mating position, 'male-to-female neck' (Squires, Norman & Stuart-Fox, 2013), and only one sperm storage organ. This organ is a highly pocketed enlargement of the distal oviduct, located within the mantle cavity, and is referred to as a bursa copulatrix (Hoving *et al.*, 2008) or a spermatheca (Norman & Lu, 1997). Males transfer spermatozoa to the female within numerous spermatophores, which most likely evert into sperm bulbs (spermatangia) within the bursa copulatrix. Females can stored this sperm for up to 145 days (Squires *et al.*, 2014). Copulation can last up to 3 h and is energetically very costly for both sexes (Franklin, Squires & Stuart-Fox, 2012). During mating and before sperm transfer, males jet water into the female's bursa copulatrix in a series of pumping movements. Female *E. tasmanica* lay a series of egg clutches over their life span (5–8 months) (Sinn & Moltschaniwskyj, 2005). Sexual selection, sperm competition, and conflict over mating frequency are therefore likely to be strong in this species and, as such, it provides an excellent opportunity to investigate the outcomes of sperm competition and sperm precedence patterns. In addition to assessing patterns of sperm precedence, we tested whether biased utilization of sperm may be attributable to male size (as a general indicator of male quality), male mating number, the duration of copulation, and the number

of pumps by the second male (potential sperm removal behaviour). By assessing paternity over the course of a female's whole laying period, we aimed to gain additional insights into temporal sperm precedence mechanisms and sperm dynamics within the female.

MATERIAL AND METHODS

DNA PROFILING

We carried out DNA profiling using five polymorphic microsatellite loci (Squires *et al.*, 2014). Primers were fluorescently labelled by adding M13 tails attached to universal ABI dyes, FAM, VIC, NED and PET, to the polymerase chain reaction (PCR) reaction, as described previously (Schuelke, 2000). DNA was extracted from whole hatchlings and from an arm tip of the laboratory males and laying females using a Qiagen QIAxtractor automated DNA extraction robot and QIAxtractor Tissue DNA protocol (QXT Tissue DNA V1). The Australian Genome Research Facility analyzed and scored fragments using an Applied Biosystems ABI3730 DNA analyzer using an LIZ-500 size standard and alleles were checked using PEAK SCANNER, version 1.0 (Applied Biosystems).

PCR volumes were 15 μl, consisting of 1 μl of extracted DNA, 1 × GoTaq Colourless Mastermix (Promega) (1 × reaction buffer, pH 8.5, 200 μM for each dNTP, 1.5 mM $MgCl_2$, 2.5 U of GoTaq DNA Polymerase), 8 pmol of reverse primer, 2 pmol of forward primer, and 8 pmol of fluorescently-labelled M13 primer. ETM700 and ETM6 amplified more reliably using GoTaq Hot Start Polymerase (Promega) in place of GoTaq Colourless Mastermix (Promega). We used three different PCR protocols (Table 1). Protocol 1 PCR conditions were 5 min at 95 °C, followed by 26 cycles of 45 s at 95 °C, 45 s starting at 64 °C and decreasing by 0.2 °C each cycle, 45 s at 72 °C, followed by 10 cycles of 45 s at 95 °C, 45 s at 58 °C, and 45 s at 72 °C, followed by 10 cycles of 45 s at 95 °C, 45 s at 57 °C, and 45 s at 72 °C, with a final step of 5 min at 72 °C. Protocol 2 PCR conditions were 5 min at 95 °C, followed by 42 cycles of 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C, with a final step of 1 min at 72 °C. Protocol 3 was identical to protocol 2, except with an annealing temperature of 48 °C. A positive control was used in each plate to account for any dye shifts or differences among plates and a negative control was used to control for contamination. In addition, at least one sample was repeated within each plate to estimate the consistency of reads.

We tested the effect of excluding primer ETM6 that deviated from Hardy–Weinberg equilibrium. However, because the results obtained were quantitatively the same, we included all five loci in subsequent analyses.

EXPERIMENTAL DESIGN

Collection, husbandry, and the experimental set-up have been described prevously (Squires *et al.,* 2012). Briefly, we collected squid using SCUBA from Phillip Bay (38°10.81′S, 144°44.60′E) in south-eastern Australia. Squid were housed in a reticulated water system at the Victorian Marine Sciences Consortium (Queenscliff) and fed live *Palaemon* sp. shrimp *ad libitum*. We housed females in the laboratory for 28 days and only those that had not laid eggs within this time were used in the experiment. This was to standardize motivation to mate and to attempt to control female mating history. However, it is possible that females had stored sperm from previous matings after 28 days (Squires *et al.*, 2012). These females (n = 12) were then mated with two different males in a design that controlled for male mating number, such that, for half the trials, females were mated first to a male that had not yet mated, and then second to a male that had previously mated once. In the remainder of the trials, females mated

Table 1. Five microsatellite loci for *Euprymna tasmanica* from Port Phillip Bay, Victoria, the protocol used and fluorescent tag

Locus	GenBank accession number	Protocol	Fluorescent tag	N_A	H_O	H_E	Hardy–Weinberg equilibrium
ETM004	KF379709	1	FAM	22	0.757	0.828	0.45
ETM006	KF379708	2	NED	15	0.730	0.707	0.005*
ETM012	KF379707	1	PET	13	0.919	0.878	0.07
ETM400	KF379710	1	PET	6	0.649	0.614	0.64
ETM700	KF379711	3	VIC	20	0.811	0.922	0.87

GenAlEx: N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; number of individuals tested for all, n = 38; none are linked and none have null alleles.

with males that had already mated once and then to a male that had not yet mated.

All adults were blotted and weighed (to an accuracy of 0.0001 g). We left females to lay eggs until senescence and collected all of the resulting hatchlings. In captivity, females lay a series of egg clutches (mean ± SD, 4.56 ± 0.34, maximum = 13) over a mean ± SD of 36.41 ± 3.65 days (maximum = 121 days). On average, egg clutches are laid 11.10 ± 0.71 days apart (range = 3–46 days) (Squires *et al.*, 2013). Tissue samples from adults and whole hatchlings were then used for genetic analysis from nine females (three of the 12 experimental females did not lay viable eggs).

Sub-sampling method

Because the number of hatchlings per female was large, for each female, we determined the number of hatchlings needed (n) to detect a 15% ($w = 0.15$) difference in paternity between male one and male two (precision estimate; Eqns 1 and 2). A 15% difference in paternity was conservatively chosen to ensure that we detected biologically meaningful differences based on those reported in other studies (Drnevich, Barnes & Siva-Jothy, 2002). We used the most conservative estimate of p (i.e. the predicted level of paternity given to one male over the other), which was at 50% ($p = 0.5$; Eqn 3). We then applied a finite population correction (where N = the true population size/total number of hatchlings per female; Eqn 4).

$$p \sim N(p, \frac{p(1-p)}{n}) \quad (1)$$

$$n \geq \frac{3.84p(1-p)}{w^2} \quad (2)$$

Eqn 3, for a 95% confidence interval:

$$\hat{p} \mp 1.96\sqrt{\frac{\hat{p}(1-p)}{n}} \quad (3)$$

$$n^* \geq \frac{N_{\mathrm{n}}}{N+n} \quad (4)$$

Once we determined the number of samples needed per female (Table 2), we sorted offspring by clutch number, and randomly chose offspring (using a random number generator) as representatives of each clutch.

Parentage analysis

We first checked our data for mismatches between offspring and their mother. This occurred in 27 out of 396 cases as a result of mis-amplification of the PCR product. These individuals were not included in subsequent analyses. There was a high level of consistency both within and between plates for genotypes (proportion of repeated alleles that were the same 0.93 ± 0.008; n = 162 repeated individuals, 50 of these more than once) and one individual was repeated in every plate to control for size standard variation among plates (within plate consistency of 0.94 ± 0.05). If, however, a repeat genotype of an individual revealed a discrepancy of more than two alleles as a result of mis-amplification, we excluded these individuals (n = 16) from the analyses. We also only included genotypes in the analyses if they amplified for more than three loci.

Paternity was assigned using a two-step process. First, the number of potential fathers was estimated using GERUD, version 2.0 (Jones, 2005) for each progeny array from each female. GERUD uses an exhaustive algorithm to reconstruct the minimum set of parents that can explain a progeny array (Jones, 2005). Briefly, GERUD starts by determining all maternal genotypes consistent with the array. The software then reconstructs potential paternal genotypes and the minimum number of sires, by subtracting the known maternal alleles. GERUD then tests these genotypes in combination with all possible maternal genotypes (Jones, 2005). We used these estimates from GERUD (i.e. of the minimum number of fathers per progeny array) as the simulation parameters in CERVUS, version 3.0 (http://cervus.software.informer.com/3.0/). CERVUS assigns paternity by using allele frequencies and a likelihood-based approach (Marshall *et al.*, 1998) and has the advantage of taking into account the effects of scoring errors and mutations in assignments.

As a result of our experimental design, in which two males were the most likely fathers, we estimated the proportion of candidate fathers sampled (for parameter settings in CERVUS) as the number of sires estimated in GERUD, divided by two. For example, if GERUD estimated that a minimum of three sires was responsible for a progeny array, the proportion of fathers sampled was set at 0.67 (we sampled two fathers so 2/3 = 0.67). In cases where GERUD estimated only two potential sires, genotypes were checked to make sure the two sampled fathers were the most likely fathers as reconstructed in GERUD. In these cases, the proportion of sampled fathers was set at one. We assumed a mistyping rate of 1%, used 10 000 iterations, 0.97 proportion of loci typed, and paternity was assigned

Table 2. The number of clutches, total number of eggs, total number of hatchlings, the estimated number of hatchlings needed to detect a 15% paternity difference and the number of hatchlings genotyped for each female

Female	Number of clutches	Total number of eggs	Total number of hatchlings	Estimated number of hatchlings needed to detect a 15% paternity difference (n)	Number of hatchlings genotyped
F1	6	180	123	31.862	33
F2	13	646	448	39.234	52
F3	7	330	250	36.689	45
F4	6	232	133	32.494	36
F5	7	293	167	34.195	39
F6	6	358	255	36.795	39
F7	3	180	49	22.902	27
F8	3	92	56	24.323	28
F9	9	384	169	34.278	38
F10	1	29	0	NA	NA
F11	0	0	0	NA	NA
F12	0	0	0	NA	NA

NA, not applicable.

at a strict confidence level of 95% (Marshall *et al.*, 1998).

Statistical analysis

Data were analyzed using R software (R Foundation for Statistical Computing) and P2 proportions (the proportion of paternity for the second male to mate) were checked for normal distribution using visual inspection of standard diagnostic plots. The only variable that needed transformation was the weight ratio between the first and second male, which we log transformed.

We tested whether P2 departed significantly from equality (0.5) for the overall dataset using one-sample *t*-tests. To determine whether paternity share changed across the whole laying period (from the first laid clutch to the last laid clutch), we ran a repeated measures analysis of variance (ANOVA) with laying period nested within female ID as the blocking factor. Because of variation among females in the number of clutches laid, the number of hatchlings per clutch, and the number of genotyped offspring per clutch, we split the whole laying period, from the first laid clutch to the last laid clutch, into two equal time periods for each female and grouped clutches within each of the two time periods. We refer to these groups as the first laying period and the last laying period. We also tested whether P2 departed significantly from equality (0.5) for each laying period separately, again using one-sample *t*-tests. We used goodness-of-fit chi-squared tests to examine whether parental contributions (P2) deviated significantly from equality for each female.

To assess the effect of recent male mating history (whether it was his first or second mating) and male mass (g) on paternity (P2), we used a general linear model (GLM) with P2 as the response variable and male mating history, male mass, and their interaction, as predictor variables. To assess the effects of copulation duration and the number of pumps by the second male on paternity (P2), we also ran a GLM. We included a weight term in each model to account for the different number of samples per female. We used the ratio of the first mating male to the second mating male for the predictor variables: male mass and copulation duration. GLMs described above were also performed on the first laying period only, given that it may represent the most ecologically relevant period (Squires *et al.*, 2014).

RESULTS

The paternity of 337 hatchlings was assessed using CERVUS, with 304 of these assigned to experimental males with 95% confidence. This left 33 offspring that are assumed to come from stored sperm. Six out of nine females showed evidence of using stored sperm, with the highest level at 0.282 for one female. However, the proportions of offspring sired from stored sperm were < 0.08 for three of these females: F3 (0.067), F5 (0.051), and F7 (0.074) (Table 3).

When considering only those offspring sired by experimental males, the proportion of paternity attributable to the last male to mate (P2) was not significantly different from 0.5 across the whole laying period (one sample *t*-test: $t = 1.606$, d.f. = 8,

Table 3. The number of offspring and proportion of paternity (in brackets) attributable to the second experimental male (P2), the first experimental male (P1) and the stored sperm

	Number (and proportion) of offspring assigned to			
Female	P2	P1	Stored sperm	Total
F1	12 (0.364)	21 (0.636)	0 (0)	33
F2	9 (0.173)	35 (0.673)	8 (0.154)	52
F3	33 (0.733)	9 (0.200)	3 (0.067)	45
F4	29 (0.806)	0 (0)	7 (0.194)	36
F5	23 (0.590)	14 (0.359)	2 (0.051)	39
F6	19 (0.487)	9 (0.231)	11 (0.282)	39
F7	14 (0.519)	11 (0.407)	2 (0.074)	27
F8	16 (0.571)	12 (0.429)	0 (0)	28
F9	36 (0.947)	2 (0.053)	0 (0)	38
Total	191	113	33	337

$P = 0.147$). However, paternal contributions did change significantly between the first and last laying period (repeated measures ANOVA: $F_{9,18} = -2.720$, $P = 0.026$). When considering the two laying periods separately, the proportion of paternity attributable to the last male to mate (P2) was significantly higher than 0.5 in the first laying period ($t = 2.746$, d.f. = 8, $P = 0.025$) but not different from 0.5 in the last laying period ($t = 0.392$, d.f. = 8, $P = 0.705$) (Fig. 1). In five out of nine females, paternal contributions deviated significantly from equality ($P < 0.0001$–0.037) (Fig. 2). In four females, sires contributed evenly to offspring: F1 ($P = 0.117$), F5 ($P = 0.150$), F7 ($P = 0.564$), and F8 ($P = 0.450$).

There was no significant interaction between recent male mating history and male mass (log ratio) (Table 4). We found no effect of recent male mating history (whether it was the male's first or second mating) or male mass (log ratio) on paternity share (both overall and for the first laying period only) (Table 4). Furthermore, there was no effect of the duration of copulation (ratio) or the number of pumps from the second male on paternity share (both overall and for the first laying period only) (Table 5).

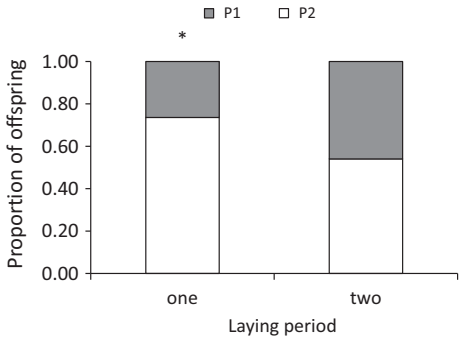

Figure 1. The proportion of paternity attributable to the first (P1) and second (P2) mating experimental males, for each laying period. Stars denotes a significant deviation from equality between P1 and P2.

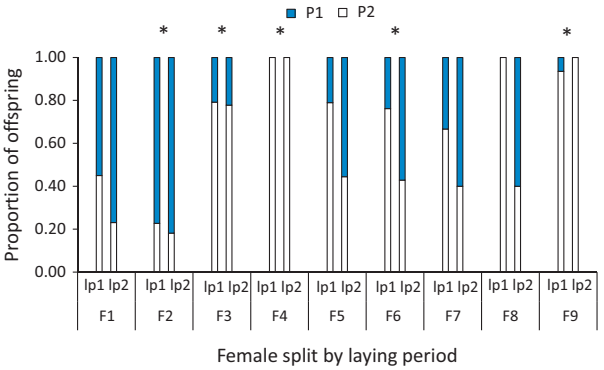

Figure 2. The proportion of paternity attributable to the first (P1) and second (P2) mating experimental males for each female (F1–F9), separated by laying period (lp1 and lp2). Stars denote a significant deviation from equality between P1 and P2 overall for female.

DISCUSSION

In a design controlling for recent mating history, mating position, and frequency, we demonstrate that the last male to mate gains a paternity advantage in dumpling squid, *E. tasmanica*, at least at the beginning of the laying period. The temporal effects of a decreasing P2 (last male advantage) may suggest that spermatangia are spatially stratified such that sperm from spermatangia of the last mating male are first to come in contact with eggs for fertilization. Alternatively, the number or velocity of sperm released from spermatangia may be temporally stratified. For example, if spermatozoa are initially released at high velocities or concentrations and, subsequently, at lower concentrations or velocities over an extended period, this would result in the observed reduction of P2 to approximately 50% over successive clutches. Furthermore, sperm displacement behaviour that increases the ratio of the number of sperm from the second male in comparison to the first male, may also contribute to last male sperm precedence. Despite the general pattern of last male sperm precedence, there was considerable variation among females in sperm use patterns, with one female showing first male sperm precedence. This variation was not attributable to any of the traits we measured, including whether it was a male's first or

Table 4. General linear model results for the proportion of paternity attributable to the second male (P2) in relation to male mating number, male mass and their interaction, for the whole laying period and for the first laying period separately

GLM Source	Whole laying period				First laying period			
	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
Male mating number	1	1.055	1.232	0.273	1	1.218	−0.558	0.601
Male mass (log ratio)	1	5.303	0.899	0.410	1	6.756	−0.495	0.641
Male mating number × male mass (log ratio)	1	3.500	−1.059	0.338	1	3.952	0.424	0.689
Error	5	15.214			5	8.709		

Table 5. General linear model results for the proportion of paternity attributable to the second male (P2) in relation to the duration of copulation and the number of pumps by the second male, for the whole laying period and for the first laying period separately

GLM Source	Whole laying period				First laying period			
	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
Copulation duration (ratio)	1	0.132	1.524	0.178	1	0.160327	0.649	0.540
Number of pumps by the second male	1	0.011	0.525	0.619	1	0.012221	0.352	0.737
Error	6	15.401			6	9.400		

second mating, mating duration, the number of pumps by the second male (i.e. sperm displacement behaviour) or male mass. Further investigation is needed to evaluate potential causes and consequences of variation among females in P2.

Last male sperm precedence is found in many taxa and is particularly common in insects (Simmons & Siva-Jothy, 1993). The usual mechanisms that explain last male sperm precedence are: (1) stratification of sperm; (2) sperm displacement; and (3) passive sperm loss (Birkhead, 2010). The sperm stratification hypothesis predicts that, because recently deposited sperm is depleted through use, sperm from earlier copulations have an unobstructed path to the egg and the proportion of P2 therefore decreases over time (Roderick *et al.*, 2003). If spermatangia are implanted in the walls of the bursa copulatrix in *E. tasmanica* and release spermatozoa over time, spatial stratification of sperm appears to be less likely than temporal stratification. Temporal stratification could result in the last mating male having a fertilization advantage only for a brief period after mating, with the proportion of P2 decreasing over time. For example, in Japanese, pygmy squid, *Idiosepius paradoxus*, sperm discharge is rapid within the first 5 min and then becomes intermittent (Sato, Kasugai & Munehara, 2014). Consistent with such temporal stratification, the proportion of P2 decreases over time from 75% to 54% in *E. tasmanica*. This reinforces the importance of collecting fertilized eggs in a temporal series to understand sperm dynamics within the female (Jones *et al.*, 2002). In another study in which females were collected from the wild and allowed to lay a series of eggs in the laboratory (Squires *et al.*, 2014), we found that sperm use did not change over time and that sperm from different males did not consistently dominate separate clutches. Taken together, these results suggest that the timing between mating and laying eggs is important, that the last mating male has an advantage if females lay eggs quickly after mating, and that this last male advantage decreases over time.

In *E. tasmanica*, the number of sires in egg clutches collected directly from the field is higher than in clutches laid sequentially in the laboratory (Squires *et al.*, 2014). This suggests either that females mate in between laying bouts (laying different egg clutches) in the field or that they mate more than twice before laying a series of eggs. Mating in between laying has been observed in other cephalopods (Arnold, 1962; Hanlon & Messenger, 1996). In *E. tasmanica*, eight out of the twelve egg clutches that we collected directly from the field (Squires *et al.*, 2014) had one sire contributing over 50% to the clutch. Although we do not have information on the mating history of the females laying these clutches, the combined results suggest that this male may have been the last mating male.

Both sperm displacement and passive sperm loss hypotheses lead to the numerical dominance of the last male's sperm and could lead to last male sperm

precedence. However, because P2 values were not significantly different from 0.5 by the end of the laying period, this shows that sperm from the first male are still present within the bursa copulatrix. This suggests that they are probably not passively lost (at least not within the time frame of the present study) and shows that sperm displacement is not complete. Sperm displacement behaviour is common in many taxa, particularly in insects (Birkhead, 2010), and can be elaborate (Gack & Peschke, 1994). A mating behaviour employed by certain cephalopods, where the males pumps or jet water towards the female's sperm storage organ, is considered to function in removing sperm from rival males stored in the spermatheca (*Sepia officinalis*: Boal, 1997; Hanlon, Ament & Gabr, 1999; *Sepia apama*: Hall & Hanlon, 2002; Naud *et al.*, 2004). Indeed, Wada *et al.* (2005) confirmed that removed debris contain live sperm in *Sepia lycidas.* Also in this species, males are able to determine the mating history of the female and increase the period of sperm removal behaviour if the stored sperm is not their own (Wada *et al.*, 2010). Male *E. tasmanica* increase the number of pumps if mating with a recently mated female but, unlike *S. lycidas*, they are unable to distinguish their own from rival spermatangia (Squires *et al.*, 2013). However, in the present study, we did not find a significant relationship between the number of pumps by the second male and paternity share. Similarly, there is no relationship between flushing duration and fertilization success in *S. apama* (Naud *et al.*, 2004).

Mate guarding is also a commonly evolved strategy for increasing paternity share, particularly in systems with last male sperm precedence (Parker, 1970), and long copulation durations may be a form of mate guarding in some species. Mate guarding is a common strategy in many cephalopods (*Loligo vulgaris reynaudii*: Hanlon, Smale & Sauer, 2002; *Abdopus aculeatus*: Huffard, Caldwell & Boneka, 2008; *S. apama*: Hall & Hanlon, 2002; *Sepioteuthis australis*: Jantzen & Havenhand, 2002). Despite not finding a significant effect of mating duration on paternity share, the long mating duration in *E. tasmanica* may still represent a form of mate guarding that is only apparent under male–male competition. However, the time needed for successful eversion of spermatophores in *E. tasmanica* might be an important factor contributing to the long copulation duration.

Where they have been examined, temporal effects are important for fertilization dynamics. For example, a study on P2 patterns in a temporal series in the rough-skinned newt, *Taricha granulosa*, revealed complete mixing of sperm within the spermatheca that would not otherwise have been apparent (Jones *et al.*, 2002). In cephalopods, the paternity share of offspring from female *Loligo pealeii* mated to two different males was dependent on the time interval between the first mating and laying the second clutch (Buresch *et al.*, 2009). If the interval is short, the first male (considered the fitter male in this design) gains significantly more paternity than if the interval is long. If spermatangia are embedded in the walls of the female's bursa copulatrix in *E. tasmanica*, the rate of spermatozoa discharge from the spermatangia could have important implications for sperm competition.

Female control of the timing of egg laying could be a potential mechanism of cryptic female choice of sperm. For example, females could bias paternity by laying eggs soon after mating with a preferred male (especially in a system with last male sperm precedence) or choose to re-mate after mating with a non-preferred male (Boorman & Parker, 1976; Simmons, 2001a). Although the timing of egg laying could be a widespread mechanism of cryptic female choice, to our knowledge, it has only been demonstrated in the soldier fly *Merosargus cingulatus* (Barbosa, 2009). It has also been suggested to occur in the Californian market squid, *Loligo opalescens*, where females increased the interval between successive laying events after a new mating (N. Kangas and R. T. Hanlon pers. comm.; Buresch *et al.*, 2009). The faster production of eggs by polyandrous *E. tasmanica* compared to females mated only once could also support this suggestion (Squires *et al.*, 2012). Monandrous female dumpling squid might be waiting to lay eggs until mating with males of sufficiently high quality or to ensure a high genetic diversity of offspring. If males are able to manipulate the timing of egg laying, such as by manipulative seminal proteins (Wolfner, 2009), this could be another effective avenue for increasing male reproductive success.

We found marked variation in P2 values among female *E. tasmanica,* with five females producing offspring that had unequal paternity (biased toward one male) and the other four producing offspring with an even mix of paternity. Variation in P2 values in *E. tasmanica* could not be explained by male mass, perhaps suggesting that male mass is not a good indicator of male quality in this species. Phenotypic plasticity is common in cephalopods (Pecl & Jackson, 2008; Storero *et al.*, 2010) and mass could be an unreliable indicator of male quality. Thus, variation in P2 could also be attributed to male traits that we did not measure. Females could bias paternity towards favourable males by controlling the timing of egg laying (by choosing to re-mate before laying eggs) or by removing unwanted spermatozoa from their bursa copulatrix (via mantle contractions or using their arms). These possibilities deserve fur-

ther investigation. Another explanation for variance in P2 among females and a potential explanation for polyandry is that females mate multiply to avoid incompatible or related genotypes; such a possibility could potentially be addressed with a larger assembly of microsatellite markers. An alternative explanation for variation in P2 among females is that it results from purely random effects of incomplete sperm mixing (Harvey & Parker, 2000) and variation in the rate and concentration of release of spermatozoa. The result that the sperm use patterns of laboratory-laying females (i.e. not given the opportunity to mate between clutches) do not change over time is consistent with the continuous release of spermatozoa from spermatangia over time (Squires *et al.*, 2014). Inter- and intraspecific variation in P2 is common in many species (Lewis & Austad, 1990; Cook, Harvey & Parker, 1997; Corley *et al.*, 2006). P2 may vary as a result of numerous factors, including male or female traits and their interaction (Wilson *et al.*, 1997). Unless specific experiments are conducted to partition variance among individual males and females separately, we cannot definitively assess the drivers.

In the present study, we found that clutches usually contain more than one sire, and thus more genetically diverse compared to clutches sired by a single male. The genetic diversity hypothesis for the evolution of polyandry proposes that genetically diverse clutches are more successful because they will have greater variation in resource use, disease resistance, and predatory defences, as well as compete less and survive better (Yasui, 1998). This might be particularly important in variable environments (Aguirre & Marshall, 2012). Experiments assessing the survival of genetically diverse clutches are needed to test this hypothesis in *E. tasmanica*. Building on the present study, experiments designed to partition variance among male and female components (and their interaction) will provide further insights into the genetic benefits hypothesis for the evolution of polyandry.

ACKNOWLEDGEMENTS

We thank Amanda Franklin, Rod Watson, and Ben Wegener for help with collections and Gordon Gully and Ana Alcaro for help with animal husbandry. We thank Jo Sumner, Adnan Moussalli, Mike Gardner, Raoul Mulder, and Sandy Clarke for help with genetic analysis. We also thank two anonymous reviewers for their helpful comments. This research was supported by funds from the Hermon Slade Foundation, Holsworth Wildlife Endowment, Museum Victoria, and the Victorian Marine Science Consortium.

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