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Multiple paternity but no evidence of biased sperm use in female dumpling squid *Euprymna tasmanica*

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ABSTRACT: Assessing the natural level of multiple paternity and sperm-use patterns provides important information regarding sperm competition and the potential for cryptic female mate choice. We developed 5 novel polymorphic microsatellites for the dumpling squid *Euprymna tasmanica* and used them to measure multiple paternity from both field- and laboratory-laid clutches. All clutches had multiple paternity, with a minimum of 2 to 4 sires per clutch, consistent with benefits to females of polyandry in this species, and indicating strong potential for sperm competition. We found a higher level of multiple paternity within field-laid eggs than in the first and second clutches from females that laid in the laboratory. The level of multiple paternity did not change over successive clutches from individual laboratory females and sperm from different males did not consistently dominate separate clutches, although individual females differed in patterns of sperm use. This suggests that spermatangia may be somewhat mixed within the female spermatheca at the time of fertilisation. Sperm competition is enhanced by high sperm longevity, and we found that viable sperm could be stored for up to 145 d in the laboratory. Overall, our results suggest strong potential for post-copulatory sexual selection in this species, due to the presence of sperm from multiple males in the female sperm storage organ competing for fertilisation of the female's eggs.

KEY WORDS: Multiple paternity · Sperm-use patterns · Sperm storage · Cephalopod

INTRODUCTION

Many species mate multiply, which generates conflict of interest between the sexes, particularly in regards to control of paternity (Chapman 2006). Consequently, males and females have evolved elaborate structures and behaviours to enable them to gain control over paternity. For example, the complex morphology of female sperm storage organs in many species may reflect selection pressure on females to bias paternity towards particular males (cryptic female choice of sperm) (Walker 1980, Birkhead et al. 1993, Keller & Reeve 1995, Eberhard 1996). Males of many species have evolved different traits that enhance paternity under sperm competition, such as elaborate sperm structures (Birkhead & Hunter 1990, Gack & Peschke 1994) and long sperm longevity within the female (Coe 1942). Despite the importance of understanding the processes of sperm competition within females, we still have little understanding of the process for many animal groups. In addition, because the effects of sperm competition are difficult to rule out experimentally, evidence for cryptic female choice of sperm remains rare (Birkhead 1998).

Investigating whether sperm use changes over time could provide a good starting point for under-

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standing paternity or sperm biasing in females (Bangham et al. 2003). For example, female soldier flies *Merosargus cingulatus* bias paternity towards males that perform copulatory courtship behaviour by ovipositing immediately after mating (Barbosa 2009). In addition, assessing sperm longevity and the level of multiple paternity in the field provides important information regarding the intensity of sperm competition and the natural level of polyandry (female multiple mating). It is particularly valuable to assess the level of multiple paternity or genetic mating system (actual paternity or maternity of offspring) for species in which the social mating system (including observable social pairings, dominance hierarchies among males and actual copulations, Hughes 1998) is difficult to quantify. This is especially true in species that have extended breeding seasons, long mating durations and need specialised equipment to be observed.

Cephalopods have the potential for strong sperm competition with promiscuous mating systems and, in some cases, complex mating dynamics (Hanlon 1996, Hall & Hanlon 2002, Iwata et al. 2005). Genetic assessment of paternity of eggs collected from the field or laid in the laboratory have revealed multiple paternity in all cephalopod species studied to date (i.e. between 2 and 5 sires within one laying bout/clutch; Shaw & Boyle 1997, Buresch et al. 2001, Emery et al. 2001, Shaw & Sauer 2004, van Camp et al. 2004, Iwata et al. 2005, Voight & Feldheim 2009). Cryptic female choice of sperm has been confirmed in 2 species of cephalopods (Hanlon & Messenger 1996, Sato et al. 2013). Cephalopod sperm are also typically remarkably long-lived (up to several months), with lifespans close to (and in some cases, beyond) the limits for free-living sperm of marine invertebrates (Naud & Havenhand 2006, Hoving et al. 2010, Rodrigues et al. 2011). This longevity, and the large number of sperm that are usually transferred within one mating, suggests that females, at least for some cephalopods, mate more times than are necessary to fertilise all their eggs (Naud & Havenhand 2006). Such sperm longevity increases the potential for sperm competition and post-copulatory sperm choice.

Euprymna tasmanica (Pfeffer, 1884) is a small semi-solitary nocturnal squid that does not have a specific breeding season. Females have a highly pocketed sperm storage organ located at the distal end of the oviduct within the mantle cavity (Norman & Lu 1997). It has been suggested this structure could facilitate cryptic female choice of sperm to bias paternity post copulation (Hanlon & Messenger 1996). Females lay a series of egg clutches (see Squires et al. 2013 for a detailed description of spawning patterns and mating behaviour) and eggs are presumably fertilised as they pass by the spermatheca through the oviduct. Mating duration is long (up to 3 h in some pairs) and energetically costly for both sexes (Franklin et al. 2012). Males transfer numerous sperm within spermatangia in one mating, and both sexes mate multiply, resulting in a high potential for sperm competition. We do not know what the natural mating frequency is or whether females re-mate between laying clutches in the field. However, we do know that mating with 2 different males confers fitness benefits to females, with evidence that polyandrous females produce eggs faster and hatchlings that are larger relative to egg mass, compared to singly mated females (Squires et al. 2012).

In this study, we developed 5 novel microsatellite markers to assess the natural level of multiple paternity in *E. tasmanica*. We assessed multiple paternity from 12 separate egg clutches collected directly from the field. To assess the temporal patterns of sperm use, we also collected females from the field and allowed them to lay eggs in the laboratory until senescence and genotyped a subset of the resulting hatchlings. We predicted that if sperm were used in discrete batches (for example, if successive clutches have different sires), this would indicate potential mechanisms for cryptic female choice of sperm. Sperm storage time was also measured from females that laid in the laboratory to assess the intensity of sperm competition and whether polyandrous females have access to viable sperm from multiple males.

MATERIALS AND METHODS

Squid and egg collection and housing

We collected 12 clutches of eggs laid in the field from unidentified females and 60 adult female *Euprymna tasmanica* (used in a larger experiment) from Port Phillip Bay (38° 10.81′ S, 144° 44.60′ E) using SCUBA. We found the egg clutches attached to the bottom of pier pylons amongst seaweed. These clutches were collected on the same day and are therefore likely to come from different females. This sample is appropriate for genetic analysis of multiple paternity and is larger than or comparable to those used in other studies (Shaw & Boyle 1997, Buresch et al. 2001, Quinteiro et al. 2011, Fitzpatrick et al. 2012). When brought to the laboratory, we housed each clutch in a separate cylindrical container that al-

lowed water flow (diameter × depth = 8 × 10 cm, volume = 0.5 l approx.), in an open water system, covered with shade cloth and kept at a constant temperature (19 ± 1°C) at the Victorian Marine Sciences Consortium in Queenscliff. We refer to these clutches as 'field-laid'. Adult squid were housed separately in individual aquaria (length × width × depth = 24 × 24 × 24 cm, volume = 13.8 l) in an open water system and fed live *Palaemon* sp. shrimp ad libitum. We checked females every second day for eggs and kept them in the laboratory until senescence (for details see Squires et al. 2012). *E. tasmanica* live for 5 to 8 mo (Sinn & Moltschaniwskyj 2005). Egg clutches were removed and housed in separate cylindrical containers as described above. We used a subset of offspring from 8 of these females for paternity analyses and refer to these clutches as 'laboratory-laid'. We estimated sperm longevity for 60 females by counting the number of days from the last opportunity for mating to the last viable clutch laid.

We checked all clutches for hatchlings every 2 d and anaesthetised and killed a subset of hatchlings for genetic analysis. To do this, we gradually introduced small amounts of 100% ethanol to a small container holding the hatchling in seawater, and once anaesthetised, we replaced seawater with ethanol for storage. We aimed to genotype at least 20% of hatchlings from field-laid eggs and at least 15% of hatchlings from each female that laid in the laboratory. We used the program BROOD (Dewoody et al. 2000) to validate the numbers we sampled post hoc. BROOD determines the minimum number of offspring needed to detect all parental alleles in each progeny array given allele frequencies for that loci set. Briefly, BROOD does this by assessing the relationship between 2 statistics: n, the number of offspring needed per clutch to detect all marker-unique gametes, and $n^{\star}$, the number of offspring per clutch needed to observe all true gametotypes (not merely those detected by available markers).

Microsatellite primer development

We developed microsatellite loci for *E. tasmanica* isolated from 2 different libraries. We extracted whole genomic DNA, from arm tips and fin tissue of adult squid collected from Port Phillip Bay, Victoria, using the Qiagen DNeasy Blood and Tissue kit (Qiagen). One library was constructed using an enrichment method as described in Guay & Mulder (2005). Briefly, extracted DNA was digested with *Sau*3A and enriched with the following oligos: AG, GT, CT, AG, TAC, GTC, CAG, CCA, AGAT, CATA and GTAT. Enriched DNA was then ligated into a pGEM-T Easy Vector (Promega) following the manufacturer's protocol. Positive clones were sequenced using an AB3730xl 96-capillary sequencer (Applied Biosystems). Sequences were imported into GeneMapper and primer pairs were designed using Primer3 (Rozen & Skaletsky 2000). A second microsatellite library was developed using next generation 454 sequencing using the methodology described by Gardner et al. (2011). In short, we sequenced one-sixteenth of a plate using the GS-FLX 454 platform (Roche). Genetic Marker Services (www.geneticmarkerservices.com) then searched the data for repeat motifs, designed primer pairs and tested these for amplification and polymorphism. We labelled all forward primers with universal 5′ M13 tails and fluorescently labelled these by adding M13 tails attached to universal Applied Biosystems (ABI) dyes FAM, VIC, NED and PET to the PCR reaction, as described by Schuelke (2000). Reverse primers from library 2 also had 5′ GTTT 'pigtails' to reduce slippage and facilitate adenylation (Brownstein et al. 1996).

We tested 15 loci across both libraries for amplification and polymorphism using 8 individual adult squid from Port Phillip Bay (38° 10.81′ S, 144° 44.60′ E). Of these loci, 8 consistently amplified using this labelling method. We then tested these loci for deviations from Hardy-Weinberg equilibrium (HWE), the presence of null alleles and linkage equilibrium on an additional 16 individual squid. We estimated deviations from HWE and basic diversity indices, including the number of alleles, the number of effective alleles, information index, observed and expected heterozygosity, and the fixation index, using GenAlEx 6.4 (Peakall & Smouse 2006). Genotypic linkage disequilibrium between all locus pairs was tested using GENEPOP 4.0.10 with 1000 steps in the Markov chain (100 batches with 1000 iterations). We tested for the presence of null alleles using the program MICRO-CHECKER (Van Oosterhout et al. 2004). Five loci showed deviations from HWE, 4 showed signs of null alleles and 2 loci were linked (one of which also contained a null allele).

Following recommendations of Jones et al. (2010), we chose a panel of 5 loci that could be multiplexed, contained no null alleles and were not linked, to be used for paternity analyses (Table 1). This panel was then tested on an additional 14 individuals (total of 38 individuals). Of these 5 loci, 1 (ETM6) showed deviations from HWE. However, results were quantitatively the same with and without this locus, so we present results of paternity analyses that include this locus.

Table 1. Characterisation of 5 microsatellite loci for *Euprymna tasmanica* from Port Phillip Bay, Victoria (GenAlEx: Na: number of alleles, Ho: observed heterozygosity, He: expected heterozygosity, number of individuals tested for all is n = 38, none are linked and none have null alleles). *locus that is out of Hardy Weinberg equilibrium (see 'Materials and methods: Microsatellite primer development')

Locus	GenBank accession number	Motif	Primer sequence (5' to 3')	Size range (bp)	Protocol	Fluorescent Tag	Na	Ho	He	HWE
ETM004	KF379709	(CA)12	F-CACGACGTTGTAAAACGACACGTAACGGTTGATATGCCG R-GTTTGGTTGCCAAGCAAGACGAG	263 – 373	1	FAM	22	0.757	0.828	0.45
ETM006	KF379708	(ATT)15	F-CACGACGTTGTAAAACGACGCAATAAGCCTCCGCAAGG R-GTTTGACGTGGAAAGCGCACTAC	196 – 241	2	NED	15	0.73	0.707	0.005*
ETM012	KF379707	(CTA)12	F-CACGACGTTGTAAAACGACTGTCTGTTTCCTTGCGACGG R-GTTTGGGCTCAAAGCTATGGATGG	199 – 241	1	PET	13	0.919	0.878	0.07
ETM400	KF379710	(GT)20	F-CACGACGTTGTAAAACGACAGCACACAAATGACATCAGAGG R-CACACACGCACTCACTTCG	349 – 385	1	PET	6	0.649	0.614	0.64
ETM700	KF379711	(CT)15	F-CACGACGTTGTAAAACGACTGTACACTTAATGACCGAAAGC R-GCCCCAGTAAACAACAGTGC	312 – 388	3	VIC	20	0.811	0.922	0.87

DNA extraction and PCR protocol

We extracted DNA from whole hatchlings and from an arm tip of the laboratory females using a QIAGEN QIAxtractor automated DNA extraction robot and the QIAxtractor Tissue DNA protocol (QXT Tissue DNA V1). Fragment analysis and scoring was undertaken by the Australian Genome Research Facility (AGRF) on an Applied Biosystems ABI3730 DNA analyser using an LIZ-500 size standard, and alleles were checked using PEAK SCANNER 1.0 (Applied Biosystems).

The final PCR volume was 15 µl, consisting of 1 µl extracted DNA, 1× GoTaq Colourless Mastermix (Promega) (1× reaction buffer pH 8.5, 200 µM of each deoxynucleoside triphosphate [dNTP], 1.5 mM $MgCl_2$, 2.5 U GoTaq DNA polymerase), 8 pmol reverse primer, 2 pmole forward primer and 8 pmol fluorescently labelled M13 primer. ETM700 and ETM6 amplified more reliably using GoTaq Hot Start Polymerase (Promega) in place of GoTaq. We used 3 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, and 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 used to control for contamination. In addition, at least one sample was repeated within each plate to estimate the consistency of reads.

Parentage analysis

We used 2 methods to determine the level of multiple paternity for each clutch. Firstly, we used a standard allele counting method in which the maternal alleles are excluded, and the remaining number of different alleles are counted and divided by 2 to get an estimate of the minimum number of sires. For this, we used only those individuals in which at least 3 loci amplified (n = 203 individuals from 12 clutches for the field-laid clutches and n = 289 individuals from 34 clutches for the laboratory-

laid eggs). We then validated these results using the program GERUD 2.0, which provides an estimate of the minimum number of sires based on allele frequencies. Briefly, GERUD 2.0 reconstructs the minimum set of parents that can explain a progeny array using an exhaustive algorithm (see Jones 2005 for more details). We assumed a mistyping rate of 1% and used 10 000 iterations, the proportion of loci typed was 0.97, paternity was assigned at a strict confidence level of 95% (Marshall et al. 1998) and we used the genotypes with the highest probability. As GERUD 2.0 cannot accommodate missing data, only those individuals that amplified across all loci were included in this analysis (n = 142 individuals from 12 clutches for the field-laid eggs and n = 223 individuals from 31 clutches for the laboratory-laid eggs; see Figs. 1 & 2). Despite this, GERUD 2.0 has the advantage of providing the likely genotype for contributing sires and can therefore be used to track different potential sires among clutches laid by the same female.

Statistical analysis

We performed statistical analyses in R (R Development Core Team 2010). All data were checked for homogeneity of variance and normality and all met these assumptions; means and SE are reported. Due to the variation in the number of clutches produced per female (mean no. of clutches: 5 ± 1.36, range: 1 to 10), we grouped clutches numbered 3 and above into 1 group and refer to these as 'Clutch 3+'. We used 2-sample *t*-tests to assess the differences in the number of sires between field and laboratory-laid eggs (all clutches combined), and for differences in the number of sires between field clutches and the first, second and third+ clutch of laboratory-laid eggs separately. We also tested for differences between the 2 methods for estimating multiple paternity (allele counting and GERUD 2.0) using a 2-sample *t*-test. To test for any effect of time (clutch number) on the number of sires in the laboratory-laid egg clutches, we ran a linear mixed model. In this model, we included female ID as a random factor to account for repeated measures of multiple clutches from the same female and hatchling number as a covariate to account for variation in the number of hatchlings among females and clutches. We used a goodness-of-fit χ^2 test to examine whether paternal contributions deviated significantly from equality for each field-collected clutch and each laboratory female.

RESULTS

Multiple paternity

The multiple paternity estimates (for the allele counting method), are based on a total of 203 individuals for the field-laid eggs (representing 44 ± 4% of all hatchlings for this group), and 289 individuals for the laboratory-laid eggs (representing 35 ± 11% of all hatchlings for this group). In all but one case, the sample sizes were above the prescribed mean ($n^\star$) sample sizes estimated in BROOD (see Appendix). This means that for all but one clutch, the estimate of the number of sires is likely to be representative of the true level of multiple paternity. The estimate for the one field-laid clutch (clutch number F2) that had a sample number below the prescribed number is a potential underestimate of the true number of sires. We therefore provide descriptive statistics with and without this clutch. Exclusion probabilities were estimated using GERUD 2.0 and were 98% for all loci combined with neither parent known for the field collected clutches, and 99% for all combined loci with one parent known for the laboratory-laid clutches. As such, these markers are suitable for future parentage assignment studies and could also be used in population genetic studies.

Estimates of the minimum number of sires from GERUD 2.0 were not statistically different from the allele counting method ($t = -0.22$, df = 38, p = 0.83). We therefore use the estimates from the allele counting method because this method included more individuals. However, because GERUD 2.0 provides a genotype for each potential sire, these can be used to track different sires among clutches. Therefore, we present results from the GERUD 2.0 analyses in Figs. 1, 2 & 3 that compare the proportion of paternity of different sires among clutches.

All field clutches, and sets of clutches from laboratory females showed multiple paternity, with a mean of 2.67 ± 0.24 sires (median: 2.5, range: 2 to 4) for field-laid egg clutches and a mean of 2.25 ± 0.18 sires (median: 2, range: 2 to 3) across clutches from each laboratory female (Table 2, Figs. 1 & 2). The average level of multiple paternity for the field-laid clutches excluding the clutch that did not conform to BROOD requirements (F2) was 2.73 ± 0.24 sires (median: 3, range: 2 to 4). The overall means for these 2 groups (field-laid clutches and clutches from each laboratory female, including F2) were not statistically different (2-sample *t*-test: $t = 1.83$, df = 18, p = 0.08). However, when considering the number of sires in the first, second and third+ clutch from laboratory females sepa-

Table 2. Total number of hatchlings of *Euprymna tasmanica*, the number of sires as estimated by the allele counting method and corresponding sample sizes (n), and the proportion of the total number of hatchlings that the sample size for each method represents for the field-laid clutches and clutches from laboratory females

Field clutch number	Total number of hatchlings	Number of sires (n)	Proportion of total hatchlings per clutch genotyped
F1	47	4 (20)	0.43
F2	22	2 (5)	0.23
F3	22	2 (16)	0.73
F4	66	4 (26)	0.39
F5	31	3 (14)	0.45
F6	62	2 (25)	0.40
F7	27	3 (16)	0.59
F8	14	2 (9)	0.64
F9	61	2 (18)	0.30
F10	35	2 (20)	0.57
F11	59	3 (17)	0.29
F12	56	3 (17)	0.30
Laboratory female	**Total number of hatchlings (number of clutches)**	**Number of sires (n)**	**Proportion of total hatchlings per clutch genotyped**
L1	51 (1)	2 (31)	0.61
L2	236 (5)	2 (37)	0.16
L3	671 (10)	2 (46)	0.07
L4	281 (7)	2 (48)	0.17
L5	268 (8)	3 (48)	0.18
L6	200 (7)	2 (44)	0.22
L7	14 (1)	3 (13)	0.93
L8	50 (1)	2 (22)	0.44

rately, we found a significant difference between the number of sires in the field-laid clutches and the number of sires in Clutch 1 (t = −2.68, df = 18, p = 0.015) and Clutch 2 (t = 2.77, df = 15, p = 0.014) and a trend for Clutch 3+ (t = −2.05, df = 15, p = 0.058) of the laboratory-laid eggs. In all cases, the mean number of sires in the field clutches was greater than in laboratory clutches (Tables 2 & 3).

Sperm-use patterns

For clutches from laboratory females, the mean number of sires did not differ among clutches ($F_{2, 7}$ = 0.61, p = 0.57). The means were 1.87 ± 0.13 for Clutch 1, 1.60 ± 0.27 for Clutch 2 and 1.80 ± 0.42 for Clutch 3+ (Table 3, Fig. 3). The paternal alleles were repeated among clutches in most cases. This was confirmed using the genotypes estimated in GERUD 2.0. These means (and those presented in the previ-

Fig. 1. Relative contribution of sires of *Euprymna tasmanica* to field-collected clutches estimated using GERUD 2.0 software. *Paternal contributions deviated significantly from equality (goodness-of-fit χ^2 tests $p < 0.05$)

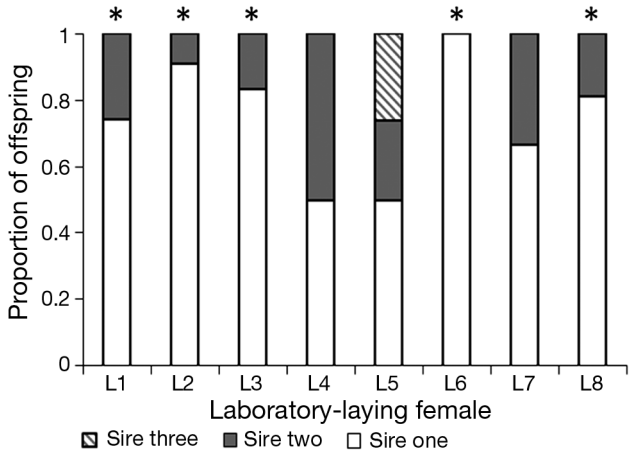

Fig. 2. Relative contribution of sires of *Euprymna tasmanica* to clutches from individual laboratory females estimated using GERUD 2.0 software. *Paternal contributions deviated significantly from equality (goodness-of-fit χ^2 tests $p < 0.05$)

Table 3. Minimum number of sires (estimated using the allele counting method) for clutches from laboratory females of *Euprymna tasmanica*

Female	Overall number of sires	Number of sires per clutch (n) Clutch 1	Clutch 2	Clutch 3+
L1	2	2 (31)	–	–
L2	2	2 (10)	2 (13)	2 (14)
L3	2	2 (14)	1 (7)	1 (25)
L4	2	2 (5)	2 (31)	2 (10)
L5	3	2 (15)	2 (9)	3 (24)
L6	2	1 (9)	1 (15)	1 (20)
L7	2	2 (9)	–	–
L8	2	2 (16)	–	–

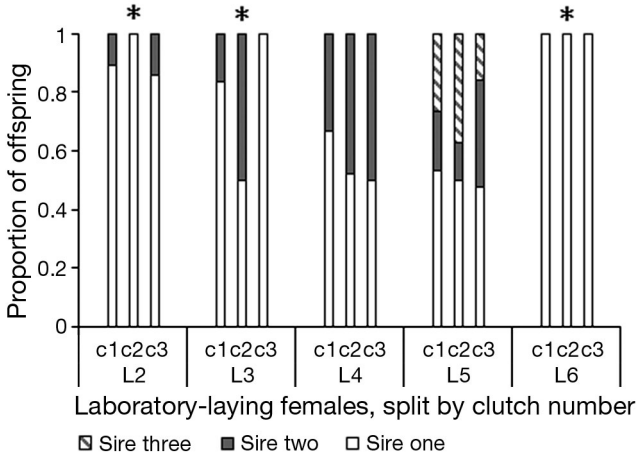

Fig. 3. Relative contribution of sires of *Euprymna tasmanica* to clutches of laboratory females, split by clutch (c), estimated using GERUD 2.0 software. *Paternal contributions deviated significantly from equality overall for that female (goodness-of-fit χ^2 tests $p < 0.05$)

ous section) are, therefore, not strictly additive. For example, Clutches 1, 2 and 3+, from laboratory-laying female 2 (L2; Table 3) all had 2 sires. As these sires were the same among all clutches, the overall number of sires was also 2.

In 4 of the 12 field-collected clutches, paternal contributions deviated significantly from equality: field clutch F1 ($p = 0.049$), field clutch F6 ($p = 0.045$), field clutch F9 ($p = 0.003$) and field clutch F12 ($p = 0.010$, Fig. 1), whereas paternity of the other 8 clutches was more mixed (goodness-of-fit χ^2 tests, $p = 0.071$ to 1; Fig. 1). In clutches from 5 out of the 8 laboratory females, paternal contributions deviated significantly from equality (goodness-of-fit χ^2 tests, $p = < 0.0001$ to 0.012; Fig. 2). Sires contributed more evenly in broods of the other 3 females: L4 ($p = 1$), L5 ($p = 0.071$) and L7 ($p = 0.317$, Fig. 2).

Sperm longevity

On average, females stored sperm for at least 56 ± 3.97 d. The maximum time between collection (or mating in the laboratory) and laying the last viable clutch was 145 d ($n = 60$). A total of 21 of these females laid viable clutches after 70 d in the laboratory. This represents a large proportion of their adult lifespan (5 to 8 mo, Sinn & Moltschaniwskyj 2005).

DISCUSSION

Our results show that clutches laid by female *Euprymna tasmanica* in the laboratory and collected from the field had multiple paternity. This demonstrates that all females mate multiply and store sperm from multiple males. We also investigated patterns of sperm use for females laying a series of clutches in the laboratory and show that levels of multiple paternity did not change over time, suggesting sperm were not being used preferentially or in discrete batches. There was a higher level of multiple paternity in the field-laid clutches than in both Clutches 1 and 2 of the laboratory-laid eggs (with a trend for this pattern in Clutch 3+). This suggests that, despite the costs associated with mating, females mate between laying clutches in the field. The potential for strong sperm competition indicated by multiple paternity in this species is reinforced by high sperm longevity, with viable sperm stored for 145 d in the laboratory.

Most clutches laid in the laboratory and collected from the field showed multiple paternity. Multiple paternity within reproductive bouts is widespread in animals (Archer & Elgar 1999, Arnqvist & Nilsson 2000, Jennions & Petrie 2000, Bretman & Tregenza 2005) and is prevalent among a range of marine organisms. High frequencies of multiple paternity (for example, 58% in Kemp's ridley turtle *Lepidochelys kempi*, Kichler et al. 1999; 71 to 100% in the intertidal crab *Petrolisthe scinctipes*, Toonen 2004; 92% in the knobbed whelk *Busycon carica*, Walker et al. 2007; 92% in the ascidian *Botryllus schlosseri*, Johnson & Yund 2007) indicate that sperm competition is a major feature of these mating systems. Assessing genetic mating systems (the level of multiple paternity) using genetic tools provides insights into the strength of sperm competition and is particularly valuable for organisms that are difficult to observe for extended periods, such as is the case for many marine animals. Studies on the genetic mating system in cephalopods examined to date have all revealed multiple paternity, with values of 2 to 5 sires within one laying bout/clutch (*Sepioteuthis australis*, van Camp et al. 2004; *Loligo pealeii*, Buresch et al. 2001; *L. forbesi*, Emery et al. 2001, Shaw & Boyle 1997; *Graneledone boreopacifica,* Voight & Feldheim 2009). The number of sires (a minimum between 2 and 4) recorded here for *E. tasmanica* fall within this range and demonstrates that all females mate multiply and store sperm from multiple males. In addition, the comparison between the level of multiple paternity under laboratory and field conditions provides additional insights into the mating system of this animal.

The general consistency of the levels of multiple paternity over time in laboratory females does not preclude biased sperm use (towards or away from

particular males), especially when females are not given the opportunity to re-mate and replenish sperm stores between clutches. A more detailed experiment is needed to test for biases in sperm use. Nevertheless, these results suggest that sperm are not being used preferentially or in discrete batches. This suggests that spermatangia may be somewhat mixed within the pockets of the female spermatheca at the time of fertilisation. Alternatively, spermatangia may be stored in separate pockets of the spermatheca but released at the same time for fertilisation. The spermatheca of female *E. tasmanica* are highly pocketed and have muscularised walls (Norman & Lu 1997). This branched morphology resembling alveoli is common to other cephalopod species (*L. pealeii,* Drew 1911; *L. vulgaris,* van Oordt 1938; *L. forbesi,* Lumkong 1992; *Idiosepius paradoxus,* Sato et al. 2010), although female *Loliginid* and *Idiosepius* store sperm rather than spermatangia. The branched morphology of sperm-storage organs is seen in many cephalopods and has prompted researchers to suggest that females could control the release of sperm from different pockets as a means of cryptic female choice of sperm (Hanlon & Messenger 1996). Despite the potential for sperm to be stored and used differentially in complex spermatheca, especially in a species that stores sperm in spermatangia such as *E. tasmanica*, we have little evidence for this mechanism in cephalopods (Sato et al. 2010). In contrast, where it has been observed, the mechanisms for cryptic female choice of sperm is via preferential spermatophore transfer to the spermatheca (Hanlon & Messenger 1996, Sato et al. 2013). Overall, therefore, our results suggest that cryptic female choice controlled by the morphology of the spermatheca is unlikely in *E. tasmanica*. It is nevertheless possible that females exercise cryptic mate choice via other mechanisms, such as through timing of egg laying or the decision when and whether to re-mate, given that laboratory laying females in this study were not given the opportunity to mate between clutches.

The higher level of multiple paternity we detected in the field-laid clutches than in clutches of laboratory-laid eggs suggests that in the field, females mate between laying clutches or at least mate more times before laying clutches than laboratory-laying females. This is despite having enough viable sperm to fertilise additional clutches as shown by laboratory females laying a series of clutches without re-mating and despite the costs associated with mating. Previous work on *E. tasmanica* has shown that mating has significant energetic costs (Franklin et al. 2012); however, females gain reproductive benefits from mating with 2 different males (Squires et al. 2012). The 'natural' levels of multiple paternity (2 to 4) shown here suggest that there may be additional benefits to be gained by mating with 2 or more males. Alternatively, females may be unable to reject mating attempts. In a captive experiment, one female Japanese pygmy squid *I. paradoxus* copulated up to 29 times, but the seminal receptacle of females is full after 8 copulations (Sato et al. 2010). This finding suggests that this particular female was unable to physically reject mating attempts under laboratory conditions, a view supported by our observations of laboratory matings in *E. tasmanica* (i.e. when the pair is confined within a small tank). Thus, mating frequency and multiple paternity may be primarily a function of encounter rates rather than under female control. However, it is likely that females have a much greater ability to reject mating attempts in the field and mating behaviours under laboratory conditions may differ from behaviours in the field.

Whilst sperm from different males may not be used in discrete batches in *E. tasmanica,* the pattern of sperm use differs among females. In some females, paternity is biased towards one male, whilst in other females the spread of paternity is more even, or mixed, among sires. This kind of variation is prevalent in genetic mating studies (Lewis & Austad 1990, Cook et al. 1997, Wilson et al. 1997, Corley et al. 2006). For example, sperm-use patterns in loggerhead sea turtles *Caretta caretta* also revealed that some clutches showed mixed paternities while others were skewed towards one sire (Zbinden et al. 2007). This variation may reflect different mating histories of females, sperm competitive abilities among males, different mating histories/mating behaviour or alternative fertilisation/mating strategies in females. Specific experiments to disentangle these effects, such as artificial insemination experiments, are needed to determine the specific mechanisms behind this variation among females.

The potential for strong sperm competition indicated by multiple paternity is reinforced by high sperm longevity, with viable sperm stored for up to 145 d in the laboratory. One potential reason for high sperm longevity is to enable females to carry male genotypes through episodes of adverse selection (Zeh et al. 1997). Levels of sperm storage for other marine organisms vary widely and have been reported to range from weeks (e.g. red whelk *Neptunea antiqua*, Power & Keegan 2001) to a year or more (e.g. onyx slipper snail *Crepidula onyx*, Coe 1942).

The sperm longevity for *E. tasmanica* is close to the ranges recorded in other cephalopods and represents

a significant proportion of their total lifespan (5 to 8 mo, Sinn & Moltschaniwskyj 2005). For example, female *Sepiola atlantica* produce viable eggs after 104 d isolation in captivity (Rodrigues et al. 2011). Sperm cells from within intact spermatophores in *Sepia apama* are still motile after 2 mo (at 4°C) (Naud & Havenhand 2006). Sperm cells diffusing out of spermatangia in this same species survived for up to 3 d, greatly exceeding the few hours commonly recorded (Naud & Havenhand 2006) and is among the highest survival times (e.g. Johnson & Yund 2004) recorded in marine invertebrates. Unpublished evidence from female *Sepia officinalis* showed that females produced fertilised eggs 5 mo after being separated from males (S. V. Boletsky 1998 pers. comm. in Hanlon et al. 1999). The longevity of sperm in some cephalopod species could be enhanced by substances produced by cells lining the spermatheca. For example, mucous cells lining the entrance to the spermatheca of *L. vulgaris* secrete a chemical that attracts sperm into the spermatheca (van Oordt 1938), and both van Oordt (1938) and Drew (1911) suggest that these mucous cells function to immobilise sperm by providing nutrients (in *L. vulgaris* and *L. pealeii* respectively). Polysaccharides synthesised and secreted in the bottom region of the seminal receptacle in *I. paradoxus* are also suggested to provide nutrients and attract and immobilise sperm (Sato et al. 2010). However, whether sperm are stored within spermatangia as in *E. tasmanica*, or not, could influence how the female reproductive tract interacts with sperm cells, as the spermatangia membrane may prevent cell-to-cell interaction. Long-term sperm storage, such as that reported here for *E. tasmanica*, enhances the opportunity for multiple paternity and increases the likelihood of interactions and competition among sperm from multiple males (Olsson et al. 1994).

Overall, the evidence we present of female storage and use of sperm from multiple males, combined with long sperm longevity, suggests that the potential for sperm competition and sexual selection is strong. However, we found little evidence that females consistently bias paternity towards particular males or use sperm in discrete batches, although more detailed experiments are required to conclusively determine the presence of cryptic female choice. These results provide the foundation on which to assess and compare sperm competition and sperm-use patterns in this species in relation to particular male and female traits, and provide insights into post-copulatory sexual selection in cephalopods more generally.

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Appendix.

The number of offspring of *Euprymna tasmanica* needed to detect all marker-unique gametes from the unshared parent of the clutch (n^* ± SD) (Dewoody et al. 2000), the number of offspring sampled (n) and whether n is greater than the mean of n^* for field collected clutches and laboratory laying females ($n > n^*$)

Field clutch number	n^* ± SD	Number genotyped (n)	$n > n^*$
F1	17.9 ± 5.39	20	Yes
F2	9.07 ± 2.65	5	No
F3	13.97 ± 3.12	16	Yes
F4	24.73 ± 7.37	26	Yes
F5	13.90 ± 3.60	14	Yes
F6	11.6 ± 3.82	25	Yes
F7	13.87 ± 3.31	16	Yes
F8	8.46 ± 2.46	9	Yes
F9	10.2 ± 4.28	18	Yes
F10	9.77 ± 3.94	20	Yes
F11	11.13 ± 3.74	17	Yes
F12	15.73 ± 5.46	17	Yes
Laboratory-laying female	n^* ± SD	Number genotyped (n)	$n > n^*$
L1	11.13 ± 3.72	31	Yes
L2	10.67 ± 3.27	37	Yes
L3	16.83 ± 7.54	46	Yes
L4	11.53 ± 5.85	48	Yes
L5	10.5 ± 3.47	48	Yes
L6	9.43 ± 2.27	44	Yes
L7	11.33 ± 3.56	13	Yes
L8	10.03 ± 3.32	22	Yes

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