# Seasonal changes in reproductive traits and paternity in the Japanese pygmy squid *Idiosepius paradoxus*

**Noriyosi Sato[1,2,*]**

[1]**Graduate School of Fisheries Science and Environmental Studies, Nagasaki University, Nagasaki, Japan**
[2]***Present address:* Oki Marine Biological Station, Shimane University, Shimane, Japan**

ABSTRACT: Sperm production in males and sperm storage in females can change over an individual's reproductive timespan. This implies that the strength of sperm competition may also change with time. The seasonal pattern of reproductive traits and paternity in the Japanese pygmy squid, which has 2 life-history types (large and small types having long and short reproductive periods, respectively), were compared among 3 reproductive periods: early and late reproductive period of the large type and middle reproductive period of the small type. Although the gonad somatic index (GSI) decreased during one of the reproductive periods in males, the number of spermatophores and the spermatophoric complex somatic index (SCSI) increased. The GSI was higher in large-type males, whereas the SCSI was higher in the small type. Multiple paternity was detected in all egg masses, and on average about 10 males were estimated to be sires in a single egg mass. Paternity numbers differed significantly between life-history types, whereas there were no significant differences between the early and late reproductive periods in the large type. These results imply that the Japanese pygmy squid is polyandrous and exposed to a very high level of sperm competition risk. Paternity numbers did not change during reproductive periods even when the reproductive traits changed between these periods. Seasonal changes in reproductive traits may have a minor effect on paternity in promiscuous animals. However, 2 life-history strategies were observed: large-type males, invested in transferring more sperm to one female, and small-type males that stored more spermatophores to copulate with many females.

KEY WORDS: Cephalopod · Microsatellites · Parentage · Multiple paternity · Seasonal change

## INTRODUCTION

Paternity analysis using molecular markers has revealed multiple paternity in many animals, in which several males sire offspring within one female's clutch or brood, demonstrating that polyandry or promiscuous mating systems are widespread in nature (Griffith et al. 2002, Uller & Olsson 2008, Coleman & Jones 2011). Post-copulatory sexual selection occurs in polyandrous and promiscuous animals through sperm competition, in which 2 or more males compete to fertilise eggs (Parker 1970, Birkhead & Møller 1998), and cryptic female choice, in which females selectively use sperm ejaculated by a favoured male for fertilisation (Thornhill 1983, Eberhard 1996). Paternity numbers indicate the strength of post-copulatory sexual selection, and the degree of paternity skew in a clutch can reveal reproductive strategies such as the existence of an alpha male and cryptic female choice. Therefore, studying paternity can reveal complexities that might remain hidden if only mating preferences or reproductive output were measured.

Although numerous studies have examined multiple paternity, few have investigated its seasonal variability (Simmons et al. 2007, Demont et al. 2011, Janssen & Baur 2015). However, physical conditions (e.g. the number and size of eggs, Gomi et al. 2008, González-Pisani & Greco 2014; sperm storage, Simmons et al. 2007; ejaculated sperm number, Birkhead 1991, Munehara & Murahana 2010) and the environment (e.g. intensity of male–male competition, Reichard et al. 2008) vary during the reproductive period. Therefore, paternity constitution within a clutch could also be expected to vary with season. For example, in tettigoniids, females store sperm derived from more males late in the breeding season than they do early in the season (Simmons et al. 2007). In addition, the intensity of sperm competition and the proportion of multiply mated females increases during the breeding season in yellow dung flies (Demont et al. 2011). These studies indicate the importance of investigating post-copulatory sexual selection over different time scales.

Multiple paternity has been found in several cephalopod species, using genetic assessment of eggs collected in the field (Buresch et al. 2001, Emery et al. 2001, Naud et al. 2004, 2016, Voight & Feldheim 2009, Squires et al. 2014). Other species provide various examples of post-copulatory sexual selection. For example, *Heterololigo bleekeri* males show alternative mating tactics; large 'consort' males attach rope-like spermatangia containing small sperm into the oviduct of the female, whereas small 'sneaker' males attach drop-like spermatangia containing larger sperm around the seminal receptacle near the mouth (Iwata et al. 2011, 2015). In some cuttlefish, males remove spermatangia previously attached by other males during copulation (Wada et al. 2005, 2010). In *Sepia apama*, it has been reported that females selectively store sperm in the seminal receptacle from attached spermatangia (Naud et al. 2005). The consumption of spermatangia in *Sepiadarium austrinum* females after copulation may be related to cryptic female choice because this behaviour results in sperm removal (Wegener et al. 2013). In cephalopods, there is seasonal variation in maturity and fecundity derived from changes in food and temperature (Smith et al. 2005, Conrath & Conners 2014). Hence, these studies demonstrate that cephalopods are useful subjects for studying seasonality in reproductive investment and paternity.

In the Japanese pygmy squid *Idiosepius paradoxus*, males perform neither courtship nor male–male agonistic behaviour to gain access to copulation (Kasugai 2000, Sato et al. 2013a) and are able to copulate with females without forming copulatory pairs. After copu-

lation, females remove more spermatangia transferred by large males than spermatangia transferred by small males (Sato et al. 2013b, 2014). Sato et al. (2017) reported that females influence paternity via sperm removal. These studies suggest that the Japanese pygmy squid is promiscuous. However, it remains poorly understood how many males sire an egg mass and it is important to analyse the paternity of egg masses collected in the field to determine the details of the mating process and the effects of sperm competition and cryptic female choice. In central Japan, the Japanese pygmy squid has 2 life histories during the year (Kasugai & Segawa 2005, Sato et al. 2008): a small body type ('small type'; dorsal mantle length (DML) 4 to 8 mm in males, 8 to 14 mm in females) that hatches from spring to early summer (March to July) and spawns soon after (June to September), and a large body type ('large type'; DML 6 to 12 mm in males, 12 to 20 mm in females) that hatches in summer, overwinters, and then spawns the following spring (March to May). Females spawn many times, at an interval of 2 d, and large-type individuals in particular continue spawning over a period of 2 mo (Kasugai 2013). The body condition and level of sperm competition of the large type varies during the long reproductive period and also differs between the 2 life-history types.

This study investigated seasonal changes in reproductive traits (indices related to the gonads, including the gonad somatic index [GSI], number and length of spermatophores in males, spawning number, and paternity) and compared these traits between the life-history types of *I. paradoxus* to elucidate their reproductive strategies.

## MATERIALS AND METHODS

### Sample collection and measurements

In total, 207 males and 129 females were collected in May, June, July, November, and December 2012, January, March, April, May, June, July, August, October, and December 2013, and March, April, May, June, and July 2014 using a hand net and SCUBA in nearshore seagrass beds in Ohmura Bay, Nagasaki, Japan (32° 52′ N, 129° 58′ E) (see Fig. A1 in the Appendix). All specimens were preserved in 10 % formalin–seawater solution after being anaesthetised with 1 % ethanol. DML and body weight (BW) were measured to the nearest 0.01 mm and 0.0001 g, respectively. The individuals were dissected, and the reproductive organs (nidamental glands and ovary in females, and spermatophoric complex organs and

testes in males) were removed and weighed to the nearest 0.0001 g. Maturation was determined by confirming the presence of spermatophores in the Needham's sac in males and ripe eggs in the oviduct in females based on Sauer & Lipiński (1990). The number of spermatophores stored in the spermatophoric sac was recorded. Ten randomly selected spermatophores were placed on a glass slide and photographed using a microscope-mounted digital camera (VB-7010; Keyence Corporation), and their lengths were measured using IMAGE-J software (NIH).

To elucidate the relationships between life-history type and reproductive characteristics, squids collected before the end of June were classified as large type and those collected after the end of June as small type based on the seasonal variation in DML. However, 2 males, one collected on 7 June 2013 and one on 25 June 2014, were classified as small and large type, respectively, because June was the transition period between the 2 types and these males did not fit the size distribution of either type. Immature males were excluded from the analyses.

In males, the GSI, spermatophoric complex somatic index (SCSI), and total GSI (TGSI) were calculated as the ratio between BW and the weight of testes (TW), that between BW and the weight of the spermatophoric complex (SCW, i.e. the spermatophore storage organ [Needham's sac] and the spermatophoric gland), and that between BW and the weight of the reproductive system (RSW, i.e. the sum of TW and SCW), respectively—GSI = (TW / BW) × 100; SCSI = (SCW / BW) × 100; TGSI = (RSW / BW) × 100. The GSI and TGSI were also calculated in females as the ratio between the weights of the ovaries (OW) or RSW (ovary, oviduct, oviducal gland, and a pair of nidamental glands) and BW, respectively (GSI = [OW / BW] × 100; TGSI = [RSW / BW] × 100).

In total, 61 complete egg masses were collected on 29 March, 12 and 26 April, 9 and 25 May, 7 and 25 June, and 12 and 19 July 2013 using SCUBA. The number of eggs in each egg mass was recorded, and 27 egg masses collected during 3 periods (12 April [defined as 'large 1'], 25 May ['large 2'], and 12 July ['small']) were transferred to an incubation aquarium (45 × 25 × 30 cm) with a closed circulation system. The larval squid were collected after hatching and fixed in ethanol for genetic analysis.

### Paternity analysis

Paternity was assessed using microsatellite analysis of 3 loci (*Himeika1*, *Himeika6*, *Himeika26*) follow-

ing Sato et al. (2017). The exclusion probability with neither parent known was calculated as 95.6% by combining the 3 primers, indicating that the microsatellite loci in the present study were sufficient to accurately reconstruct parentage. Genotyping was conducted using 655 larval squid that had hatched from 744 eggs. DNA was extracted using a KAPA Express Extract Kit (KAPA Biosystems) according to the supplier's protocol. Polymerase chain reaction (PCR) was performed using a fluorescent primer 5'-labeled with NED, ROX, or FAM (Applied Biosystems), and the size of the PCR fragments was determined using a genetic analyser (3730xl; Applied Biosystems). The PCR mixes contained BIOTAQ PCR buffer, 50 µM of each dNTP, 1.25 mM MgCl$_2$, 1 unit of BIOTAQ polymerase (BioLine), 0.5 µM of each primer, 15 ng template DNA, and distilled water to a final volume of 10 µl. Thermal cycling reactions consisted of an initial 60 s at 94°C, followed by 25 to 30 cycles of 30 s at 94°C, 60 s at the primer-specific annealing temperature (Sato et al. 2017), and 60 s at 72°C.

Parentage assignment was analysed using the software programmes GERUD v.2.0 (Jones 2005) and COLONY v.2.0.5.8 (Jones & Wang 2010). GERUD and COLONY infer the minimum estimate and the most likely number of fathers, respectively. Paternity skew was calculated from the data of offspring assignment using COLONY and expressed as the sum of the squared proportions of offspring sired by each male (Boomsma & Sundström 1998).

### Statistical analysis

Generalised linear models (GLMs) were used to analyse whether GSI, TGSI, spermatophore number, and spermatophore length were related to DML and life-history type. The gamma distribution with log link function was used for analysing GSI, TGSI, and spermatophore length, and the negative binomial distribution with log link function was used for spermatophore number. GLMs with a Poisson distribution and log link function were used to analyse the relation between paternity number and reproductive period (large 1, large 2, and small). The egg number per clutch was used as an offset variable. Tukey's test (multcomp package) was applied to assess differences among the periods. The significance of fixed factors on dependent variables was assessed using a likelihood ratio test (LRT). All analyses were performed in R v.2.15.2 (R Development Core Team 2015).

## RESULTS

### Seasonal variability in reproductive characteristics

Japanese pygmy squid were collected from March to July; none were collected from August to February (see Fig. A1 in the Appendix). Most individuals of both sexes had matured during the collection period: 197 of 207 (95.2%) males and 124 of 129 (96.1%) females.

Only data collected in 2013 were used to determine the seasonal variability in DML and reproductive traits. DML increased from mid-March to mid-June, and decreased at the end of June (Fig. 1). In males, the GSI decreased from mid-March to mid-June, increased at the end of June, and then decreased again to mid-July, whereas SCSI showed a trend opposite to that of GSI. The TGSI in males did not vary over time, whereas the GSI and TGSI in females increased over time (Fig. 1). The number of spermatophores increased from mid-March to mid-June, and decreased at the end of June (Fig. 2A). Spermatophore length remained unchanged until mid-June, and then decreased at the end of June (Fig. 2B). The egg number per egg mass increased until the beginning of June, and decreased at the end of June (Fig. 3), which suggests that egg generation changed during June.

### Relationships between reproductive characteristics and life-history type, and between DML and life-history type

The GSI and TGSI in males were significantly lower in the small type than in the large type (GLM with LRT: GSI, $\chi^2 = 1.474$, p < 0.001; TGSI, $\chi^2 = 0.295$, p < 0.001) and decreased with DML (GLM with LRT: GSI, $\chi^2 = 0.922$, p < 0.001; TGSI, $\chi^2 = 0.219$, p < 0.01; Fig. 4A,B). There was a significant interaction between DML and life-history type in the GSI, but no significant interaction was observed between DML and life-history type in the TGSI (GLM with LRT: GSI, $\chi^2 = 0.178$, p < 0.05; TGSI, $\chi^2 = 0.042$, p = 0.19). The SCSI was significantly higher in the small type (GLM with LRT: $\chi^2 = 0.549$, p < 0.01) and increased with DML (GLM with LRT: $\chi^2 = 0.282$, p < 0.05; Fig. 4C), whereas there was no significant interaction in the SCGI model (GLM with LRT: $\chi^2 = 0.068$, p = 0.29). In females, the GSI and TGSI increased with DML (GLM with LRT: GSI, $\chi^2 = 1.662$, p < 0.001; TGSI, $\chi^2 = 2.316$, p < 0.001; Fig. 4D,E). The GSI and TGSI in the small type were significantly lower than in the large type (GLM with LRT: GSI, $\chi^2 = 1.723$, p < 0.001; TGSI, $\chi^2 = 2.461$, p < 0.001; Fig. 4D,E), and there was a significant interaction between DML and life-history type (GLM with
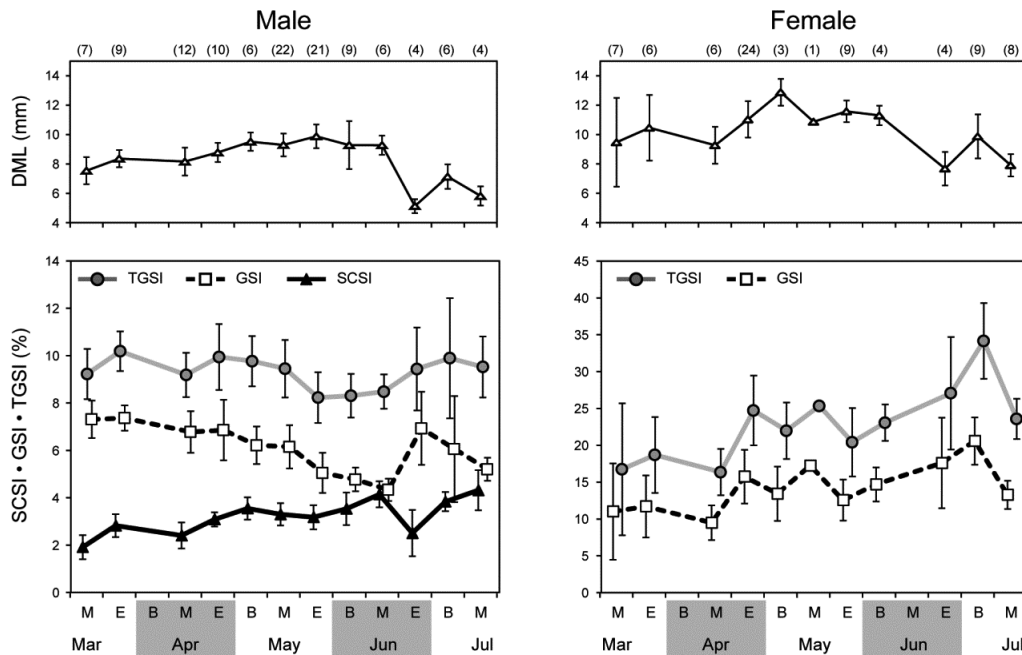


Fig. 1. Mean (±SD) seasonal variation in Japanese pygmy squid dorsal mantle length (DML), reproductive indices in males (left column), and females (right column). Sample size for each month is shown above the graphs. The letters B, M, and E on the *x*-axis indicate beginning, middle, and end of month, respectively. GSI: gonad somatic index; TGSI: total GSI; SCSI: spermatophoric complex somatic index. Note differences in *y*-axis scales
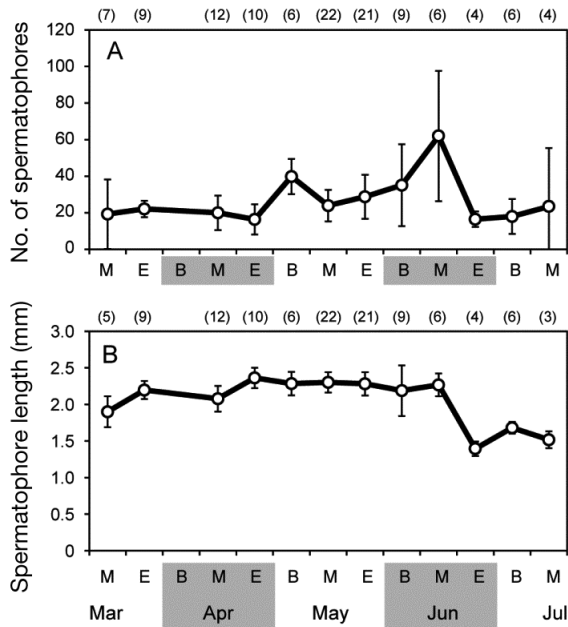
Fig. 2. Mean (±SD) seasonal variation in Japanese pygmy squid (A) spermatophore number and (B) length. Sample size for each month is shown above the graphs. The letters B, M, and E on the x-axis indicate beginning, middle, and end of month, respectively
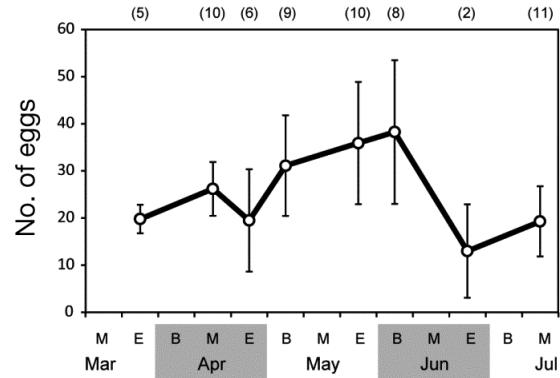


Fig. 3. Mean (±SD) seasonal variation in Japanese pygmy squid egg number in egg masses collected in the field. Sample size for each month is shown above the graphs. The letters B, M, and E on the x-axis indicate beginning, middle, and end of month, respectively

LRT: GSI, $\chi^2$ = 0.355, p < 0.05; TGSI, $\chi^2$ = 0.453, p < 0.01; Fig. 4D,E). The number of spermatophores was not significantly correlated with type, DML,

or the relationship between type and DML (GLM with LRT: type, $\chi^2$ = 0.660, p = 0.42; DML, $\chi^2$ = 2.631, p = 0.10; interaction, $\chi^2$ = 2.108, p = 0.15; Fig. 5A), while spermatophore length increased with DML and was longer in the large type (GLM with LRT: DML, $\chi^2$ = 0.533, p < 0.001; type, $\chi^2$ = 0.239, p < 0.001; Fig. 5B). There was no significant interaction between DML and life-history type (GLM with LRT: $\chi^2$ = 0.002, p = 0.48).
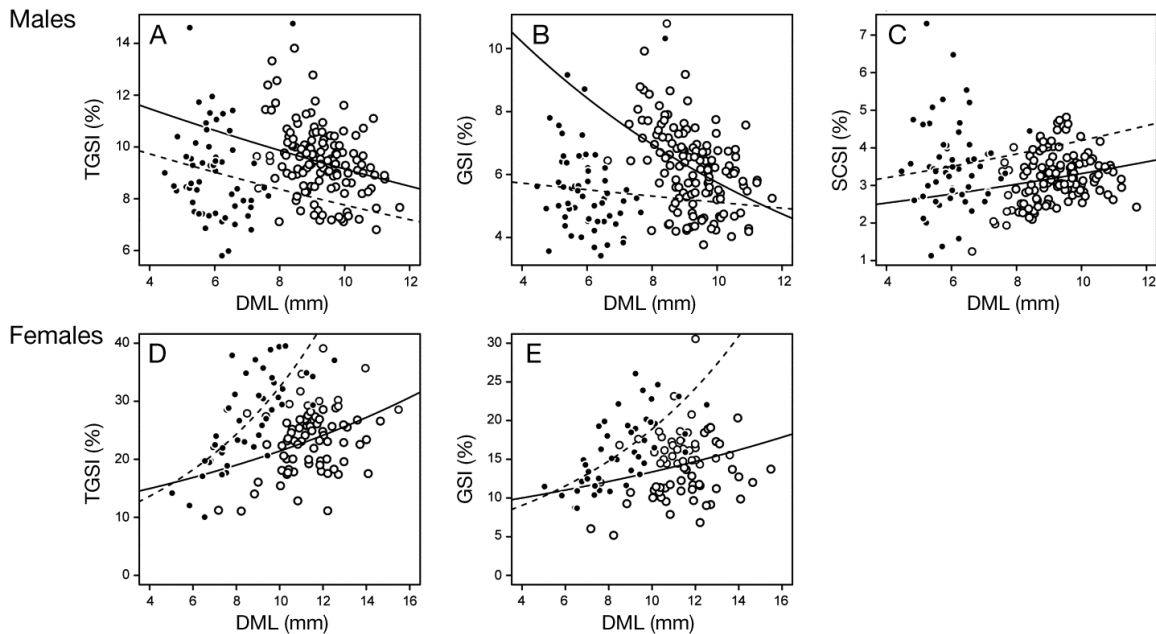


Fig. 4. Relationships between Japanese pygmy squid dorsal mantle length (DML) and (A) total gonad somatic index (TGSI), (B) gonad somatic index (GSI), and (C) spermatophoric complex somatic index (SCSI) in males, and (D) TGSI and (E) GSI in females. Filled and open circles: small and large life-history types, respectively. Solid and broken lines represent the gamma regression
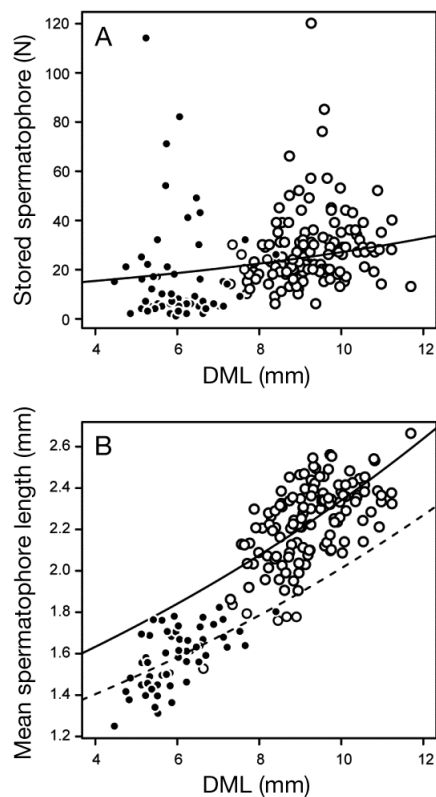
Fig. 5. Relationships between Japanese pygmy squid dorsal mantle length (DML) and (A) number of spermatophores, and (B) mean spermatophore length. Filled and open circles: small and large life-history types, respectively. Solid and broken lines represent the gamma regression

## Paternity analysis among 3 reproductive periods

Multiple paternity was detected in all egg masses. In GERUD, the estimated minimum number of fathers per egg mass was >6 in many of the egg masses. Unfortunately, the correct minimum number was not calculated because the GERUD programme was not used to calculate the paternity of egg masses with >6 fathers. COLONY estimated 11 males (SD = 1.8, range = 7 to 13) in large 1 egg masses, 12.8 males (SD = 4.1, range = 7 to 19) in large 2, and 8.9 males (SD = 2.3, range = 6 to 14) in small (Table 1). There was significant variation in these values among collection dates (GLM with LRT: $\chi^2$ = 6.613, p < 0.05). There were no significant differences between large 1 and large 2 (GLM with Tukey's test: $z$ = 1.793, p = 0.17), or between large 1 and small (GLM with Tukey's test: $z$ = 0.717, p = 0.75), but the difference was significant between large 2 and small (GLM with Tukey's test: $z$ = 2.429, p < 0.05). The proportions of the dominant sire's offspring per egg mass were 25.5% (SD = 9.9,

range = 13.6 to 45.5) in large 1, 30.6% (SD = 10.3, range = 15.8 to 45.0) in large 2, and 26.4% (SD = 4.7, range = 20.0 to 33.3) in small. The skewness of paternity was fairly low in all seasons, but the paternity pattern in one egg mass (ID L2F3) significantly differed from uniform distribution (Table 1).

## DISCUSSION

The data showed multiple paternity in the *Idiosepius paradoxus* egg masses collected from the field. The mean paternity numbers of the egg masses calculated in COLONY was 11 to 12 in the large type, and 9 in the small type. GERUD estimated a minimum of >6 fathers clutch$^{-1}$ in many of the egg masses. Although there have been several studies of paternity in cephalopod egg masses collected from the field (*Loligo reynaudii*, Shaw & Sauer 2004, Naud et al. 2016; *L. pealeii*, Buresch et al. 2001; *L. forbesi*, Shaw & Boyle 1997, Emery et al. 2001; *Euprymna tasmanica*, Squires et al. 2014; *Octopus vulgaris*, Quinteiro et al. 2011; *O. minor*, Bo et al. 2016; *Graneledone boreopacifica*, Voight & Feldheim 2009), the maximum number of fathers was not >5 in those species. Compared to these studies, the results of the present study suggest that *I. paradoxus* has a mating process involving higher promiscuity. This is supported by the values for the GSI. The GSI of *I. paradoxus* males was 4 to 7%, which is higher than in other cephalopod species (1.03 to 2.53% in *Sepiola intermedia*, 0.55 to 1.89% in *S. robusta*, 0.22 to 2.23% in *S. steenstrupiana*, Salman & Önsoy 2004; 1 to 2% in *L. reynaudii*, Olyott et al. 2006; and <1% in *O. vulgaris*, Oosthuizen & Smale 2003).

Although the TGSI did not vary during the reproductive periods of either life-history type in males, the GSI decreased and SCSI increased. Males may make a greater effort to produce sperm at the beginning of the reproductive period, and then shift their effort to making and storing spermatophores at the end of the period. It is possible that males employ 2 tactics at the end of the reproductive season: their copulation strategy is to transfer more spermatophores or to copulate with many females. However, paternity numbers and skewness did not vary between the early and late season in the large type, and if males had copulated with many females, there would have been a higher paternity number late in the season. Hence, the males may employ the latter tactic of copulating with many females, otherwise their fertilisation success would be reduced in the environment with stronger sperm competition.

Table 1. Summary of paternity analysis results for wild-caught Japanese pygmy squid egg masses in 3 reproductive periods. Percentage of embryos sired by dominant and subdominant males were assigned by COLONY. Fisher's exact test p-value is a significance of departure from uniform distribution. **Bold**: significant difference (p < 0.05). Egg masses collected on 12 April, 25 May, and 12 July 2013 were defined 'large 1', 'large 2', and 'small', respectively

| | Egg mass ID | Egg number | Paternity number by GERUD | Paternity number by COLONY | % Embryos sired by dominant male | % Embryos sired by subdominant males | Skewness | Fisher's exact test p-value |
|---|---|---|---|---|---|---|---|---|
| Large 1 | L1F1 | 32 | 6 | 12 | 31.3 | 21.9 | 0.176 | 0.68 |
| | L1F2 | 25 | >6 | 12 | 16.0 | 12.0 | 0.104 | 0.99 |
| | L1F3 | 27 | >6 | 12 | 29.6 | 11.1 | 0.141 | 0.99 |
| | L1F4 | 17 | 6 | 11 | 17.6 | 11.8 | 0.107 | 0.99 |
| | L1F5 | 19 | 6 | 13 | 15.8 | 15.8 | 0.097 | 0.99 |
| | L1F6 | 33 | >6 | 12 | 45.5 | 9.1 | 0.240 | 0.39 |
| | L1F7 | 17 | 5 | 10 | 17.6 | 17.6 | 0.128 | 0.99 |
| | L1F8 | 26 | 6 | 10 | 30.8 | 15.4 | 0.160 | 0.70 |
| | L1F9 | 8 | 4 | 7 | 25.0 | 12.5 | 0.156 | 0.99 |
| | Ave ± SD | 22.7 ± 8.1 | | 11.0 ± 1.8 | 25.5 ± 9.9 | 14.1 ± 3.9 | 0.145 ± 0.04 | |
| Large 2 | L2F1 | 20 | 4 | 7 | 45.0 | 20.0 | 0.270 | 0.26 |
| | L2F2 | 34 | >6 | 14 | 20.6 | 14.7 | 0.109 | 0.99 |
| | L2F3 | 46 | 2 | 11 | 34.8 | 26.1 | 0.221 | **0.04** |
| | L2F4 | 24 | 5 | 8 | 37.5 | 20.8 | 0.219 | 0.68 |
| | L2F5 | 53 | >6 | 19 | 37.7 | 7.5 | 0.171 | 0.12 |
| | L2F6 | 26 | >6 | 13 | 26.9 | 15.4 | 0.133 | 0.99 |
| | L2F7 | 37 | >6 | 14 | 18.9 | 16.2 | 0.112 | 0.82 |
| | L2F8 | 19 | 6 | 11 | 15.8 | 10.5 | 0.108 | 0.99 |
| | L2F9 | 44 | >6 | 18 | 13.6 | 11.4 | 0.081 | 0.99 |
| | Ave ± SD | 33.7 ± 12.2 | | 12.8 ± 4.1 | 30.6 ± 10.3 | 16.0 ± 6.0 | 0.158 ± 0.07 | |
| Small | SF1 | 18 | 6 | 10 | 33.3 | 16.7 | 0.173 | 0.91 |
| | SF2 | 12 | 3 | 7 | 25.0 | 16.7 | 0.167 | 0.99 |
| | SF3 | 18 | 6 | 10 | 22.2 | 16.7 | 0.130 | 0.98 |
| | SF4 | 10 | 4 | 6 | 30.0 | 20.0 | 0.200 | 0.99 |
| | SF5 | 17 | 5 | 9 | 29.4 | 11.8 | 0.156 | 0.98 |
| | SF6 | 10 | 4 | 8 | 20.0 | 20.0 | 0.140 | 0.99 |
| | SF7 | 13 | 4 | 8 | 23.1 | 23.1 | 0.160 | 0.99 |
| | SF8 | 16 | 5 | 8 | 31.3 | 18.8 | 0.180 | 0.93 |
| | SF9 | 34 | >6 | 14 | 23.5 | 17.6 | 0.125 | 0.92 |
| | Ave ± SD | 16.4 ± 7.3 | | 8.9 ± 2.3 | 26.4 ± 4.7 | 17.9 ± 3.1 | 0.159 ± 0.02 | |

The increases in the TGSI and GSI in females up to the end of June were consistent with the trend of increasing egg number per egg mass in the large type (Figs. 1 & 3). The females invested their energy in reproductive traits from the beginning of maturation. They may become more selective in mate choice with time because their fecundity is high late in the season. This is consistent with the results of Sato et al. (2013b), who showed that females who had previously copulated made a greater effort to remove spermatangia than did virgin females. However, females, via cryptic female choice, may also employ tactics to keep genetic bet-hedging and attempt to copulate with various males who equalise their sperm volume, because there were no differences in paternity number between the early and late season in the large type. There was a mismatch between both indices and egg number in July. Females were unable to produce enough eggs in the small type, even if they invested more in reproductive traits.

The TGSI and GSI in males were higher in the large type than in the small type, whereas the SCSI was higher in the small type. Although there were no significant differences in the number of stored spermatophores between the 2 life-history types, spermatophore length was longer in the large type. These results suggest that large-type males invest in producing fewer, longer spermatophores with more spermatozoa. It may be more important for males of the large type to transfer large numbers of sperm in a single copulation than to keep multiple reserves of spermatophores to copulate with many females. This prediction is consistent with the prediction of seasonal change in the male's tactics. Small-type males invested more in storing spermatophores than in having longer spermatophores, which indicates that their reproductive strategy may be suitable to copulate with many females. There were no differences in sperm storage number between large and small types, and both TGSI and GSI were lower in the small type than in the large type. In the small type, in which sperm competition risk is lower, males may not have to transfer more sperm to a female to secure their fertilisation. However, small-type males may have to invest more in sperm storage to achieve sufficient fertilisation in promiscuous mating systems.

The TGSI and GSI in females were also lower in the large type than in the small type. Small-type females lay smaller and fewer eggs than large-type females (Kasugai 2013). The mean egg number produced during the reproductive period was 853 in the large type and 342 in the small type. However, small-type females spawn more frequently and the spawning span is shorter (Kasugai 2013). This suggests that the investment by small-type females is to produce eggs as rapidly as possible.

Most egg masses showed low paternity skew. A last male copulating in a male-parallel position fertilised the majority of egg clutches in *Heterololigo bleekeri* (Iwata et al. 2005). Although there was no last-male precedence, fertilisation success was skewed in *Euprymna tasmanica* (Squires et al. 2015). However, *I. paradoxus* did not appear to display sperm precedence. In this species, when a female copulated with 2 successive males, the fertilisation success of the second male was correlated with the relative sperm volume of spermatangia remaining on the female's body after spermatangia removal (Sato et al. 2017). This means that sperm were transferred to the seminal receptacle from the spermatangia, where they were mixed and stored. Therefore, fertilisation success was reflected by the sperm volume of each male. This suggests that no single male transferred significantly more than others, and females did not bias paternity toward specific males.

Kasugai & Segawa (2005) reported that Japanese pygmy squid occur in nearshore areas in Nagoya, central Japan, in all months except between August and February. The squid are harvested for only 4 mo, from September to December, in the northern limits of their distribution, because they do not survive the low temperatures in winter (Sato et al. 2009). However, in Nagasaki, their seasonal disappearance is not related to low temperatures as the average winter water temperature is >6°C (N. Sato pers. obs.), which is high enough for them to survive the winter (Sato et al. 2009, Sato & Munehara 2013c). The seasonal pattern of body size during the reproductive period is similar in Nagasaki and Nagoya. Although the distribution area of immature squid can differ between the Nagasaki and Nagoya populations in the large type due to the absence of the Nagasaki population during winter, the life-cycle patterns are similar in the 2 locations. The absence of the Nagasaki population may be related to the distribution and abundance of prey.

In conclusion, the results imply that *I. paradoxus* is exposed to a very high level of sperm competition risk. However, although paternity numbers differed between life-history types (large 2 and small), there were no significant differences between the early and late periods of the large type. Male *I. paradoxus* copulate with many females during the reproductive period because they do not form pair-bonds, and copulate freely with females (Kasugai 2000), i.e. they have a promiscuous mating process. Therefore, no

variation in paternity may be observed even if the reproductive traits vary with time. However, the finding of a seasonal pattern in the reproductive traits suggests that large-type males invest in transferring more sperm to one female, and small-type males store more spermatophores to copulate with many females. It is unclear why the squid exhibited a high level of multiple paternity and did not show any skewness in their fertilisation success. To elucidate the evolutionary process of the promiscuity and the reproductive strategy in *I. paradoxus*, it is necessary to determine the benefits of promiscuity and multiple paternity.

LITERATURE CITED

Birkhead TR (1991) Sperm depletion in the Bengalese finch, *Lonchura striata.* Behav Ecol 2:267–275

Birkhead TR, Møller AP (1998) Sperm competition and sexual selection. Academic Press, Cambridge, MA

Bo QK, Zheng XD, Gao XL, Li Q (2016) Multiple paternity in the common long-armed octopus *Octopus minor* (Sasaki, 1920) (Cephalopoda: Octopoda) as revealed by microsatellite DNA analysis. Mar Ecol 37:1073–1078

Boomsma JJ, Sundström L (1998) Patterns of paternity skew in *Formica* ants. Behav Ecol Sociobiol 42:85–92

Buresch KM, Hanlon RL, Maxwell MR, Ring S (2001) Microsatellite DNA markers indicate a high frequency of multiple paternity within individual field-collected egg capsules of the squid *Loligo pealeii.* Mar Ecol Prog Ser 210:161–165

Coleman SW, Jones AG (2011) Patterns of multiple paternity and maternity in fishes. Biol J Linn Soc 103:735–760

Conrath CL, Conners ME (2014) Aspects of the reproductive biology of the North Pacific giant octopus (*Enteroctopus dofleini*) in the Gulf of Alaska. Fish Bull 112:253–260

Demont M, Buser CC, Martin OY, Bussière LF (2011) Natural levels of polyandry: differential sperm storage and temporal changes in sperm competition intensity in wild yellow dung flies. Funct Ecol 25:1079–1090

Eberhard WG (1996) Female control: sexual selection by cryptic female choice. Princeton University Press, Princeton, NJ

Emery AM, Wilson IJ, Craig S, Boyle PR, Noble LR (2001) Assignment of paternity groups without access to parental genotypes: multiple mating and developmental plasticity in squid. Mol Ecol 10:1265–1278

Gomi T, Kimura T, Hirose S (2008) Seasonal changes in the reproductive and life-history traits of *Chrysomela populi* L.(Coleoptera: Chrysomelidae). Entomol Sci 11:31–38

González-Pisani X, Greco LL (2014) Comparative reproductive effort and fecundity in the spider crabs, *Leurocyclus*

*tuberculosus* and *Libinia spinosa* (Majoidea, Brachyura). Zool Sci 31:244–250

Griffith SC, Owens IPF, Thuman KA (2002) Extra pair paternity in birds: a review of interspecific variation and adaptive function. Mol Ecol 11:2195–2212

Iwata Y, Munehara H, Sakurai Y (2005) Dependence of paternity rates on alternative reproductive behaviors in the squid *Loligo bleekeri.* Mar Ecol Prog Ser 298:219–228

Iwata Y, Shaw P, Fujiwara E, Shiba K, Kakiuchi Y, Hirohashi N (2011) Why small males have big sperm: dimorphic squid sperm linked to alternative mating behaviours. BMC Evol Biol 11:236

Iwata Y, Sakurai Y, Shaw P (2015) Dimorphic sperm-transfer strategies and alternative mating tactics in loliginid squid. J Molluscan Stud 81:147–151

Janssen R, Baur B (2015) Seasonal effects on egg production and level of paternity in a natural population of a simultaneous hermaphrodite snail. Ecol Evol 5:2916–2928

Jones AG (2005) GERUD 2.0: a computer program for the reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents. Mol Ecol Notes 5:708–711

Jones OR, Wang J (2010) COLONY: a program for parentage and sibship inference from multilocus genotype data. Mol Ecol Resour 10:551–555

Kasugai T (2000) Reproductive behavior of the pygmy cuttlefish *Idiosepius paradoxus* in an aquarium. Venus 59:37–44

Kasugai T (2013) Study of the life history of the Japanese pygmy squid, *Idiosepius paradoxus* in the Central Honshu, Japan. PhD thesis, Tokyo University of Marine Science and Technology

Kasugai T, Segawa S (2005) Life cycle of the Japanese pygmy squid *Idiosepius pardoxus* (Cephalopoda: Idiosepiidae) in the *Zostera* beds of the temperate coast of Central Honshu, Japan. Phuket Mar Biol Cent Res Bull 66:249–258

Munehara H, Murahana H (2010) Sperm allocation pattern during a reproductive season in the copulating marine cottoid species, *Alcichthys alcicornis.* Environ Biol Fishes 88:323–331

Naud MJ, Hanlon RT, Hall KC, Shaw PW, Havenhand JN (2004) Behavioural and genetic assessment of reproductive success in a spawning aggregation of the Australian giant cuttlefish, *Sepia apama.* Anim Behav 67:1043–1050

Naud MJ, Shaw PW, Hanlon RT, Havenhand JN (2005) Evidence for biased use of sperm sources in wild female giant cuttlefish (*Sepia apama*). Proc R Soc B 272:1047–1051

Naud MJ, Sauer WHH, McKeown NJ, Shaw PW (2016) Multiple mating, paternity and complex fertilisation patterns in the chokka squid *Loligo reynaudii.* PLOS ONE 11:e0146995

Olyott LJH, Sauer WHH, Booth AJ (2006) Spatio-temporal patterns in maturation of the chokka squid (*Loligo vulgaris reynaudii*) off the coast of South Africa. ICES J Mar Sci 63:1649–1664

Oosthuizen A, Smale MJ (2003) Population biology of *Octopus vulgaris* on the temperate south-eastern coast of South Africa. J Mar Biol Assoc UK 83:535–541

Parker GA (1970) Sperm competition and its evolutionary consequences in the insects. Biol Rev Camb Philos Soc 45:525–567

Quinteiro J, Baibai T, Oukhattar L, Soukri A, Seixas P, Rey-Mendez M (2011) Multiple paternity in the common

octopus *Octopus vulgaris* (Cuvier, 1797), as revealed by microsatellite DNA analysis. Molluscan Res 31:15–20

R Development Core Team (2015) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Reichard M, Smith C, Bryja J (2008) Seasonal change in the opportunity for sexual selection. Mol Ecol 17:642–651

Salman A, Önsoy B (2004) Analysis of fecundity of some bobtail squid of the genus *Sepiola* (Cephalopoda: Sepiolida) in the Aegean Sea (eastern Mediterranean). J Mar Biol Assoc UK 84:781–782

Sato N, Munehara H (2013c) The possibility of overwintering by *Idiosepius paradoxus* (Cephalopoda: Idiosepiidae) at the northern limits of its distribution. Am Malacol Bull 31:101–104

Sato N, Kasugai T, Munehara H (2008) Estimated life span of the Japanese pygmy squid, *Idiosepius paradoxus* from statolith growth increments. J Mar Biol Assoc UK 88: 391–394

Sato N, Awata S, Munehara H (2009) Seasonal occurrence and sexual maturation of Japanese pygmy squid (*Idiosepius paradoxus*) at the northern limits of their distribution. ICES J Mar Sci 66:811–815

Sato N, Yoshida M, Fujiwara E, Kasugai T (2013a) High-speed camera observations of copulatory behavior in *Idiosepius paradoxus*: function of the dimorphic hectocotyli. J Molluscan Stud 160:553–561

Sato N, Kasugai T, Munehara H (2013b) Sperm transfer or spermatangia removal: postcopulatory behaviour of picking up spermatangium by female Japanese pygmy squid. Mar Biol 160:553–561

Sato N, Kasugai T, Munehara H (2014) Female pygmy squid cryptically favour small males and fast copulation as observed by removal of spermatangia. Evol Biol 41: 221–228

Sato N, Yoshida M, Kasugai T (2017) Impact of cryptic female choice on insemination success: larger sized and longer copulating male squid ejaculate more, but females influence insemination success by removing spermatangia. Evolution 71:111–120

Sauer WH, Lipiński MR (1990) Histological validation of morphological stages of sexual maturity in chokker squid *Loligo vulgaris reynaudii* D'Orb (Cephalopoda: Loliginidae). S Afr J Mar Sci 9:189–200

Shaw PW, Boyle PR (1997) Multiple paternity within the brood of single females of *Loligo forbesi* (Cephalopoda: Loliginidae), demonstrated with microsatellite DNA markers. Mar Ecol Prog Ser 160:279–282

Shaw PW, Sauer WHH (2004) Multiple paternity and complex fertilisation dynamics in the squid *Loligo vulgaris reynaudii.* Mar Ecol Prog Ser 270:173–179

Simmons LW, Beveridge M, Kennington WJ (2007) Polyandry in the wild: temporal changes in female mating frequency and sperm competition intensity in natural populations of the tettigoniid *Requena verticalis.* Mol Ecol 16:4613–4623

Smith JM, Pierce GJ, Zuur AF, Boyle PR (2005) Seasonal patterns of investment in reproductive and somatic tissues in the squid *Loligo forbesi.* Aquat Living Resour 18: 341–351

Squires ZE, Wong BBM, Norman MD, Stuart-Fox D (2014) Multiple paternity but no evidence of biased sperm use in female dumpling squid *Euprymna tasmanica.* Mar Ecol Prog Ser 511:93–103

Squires ZE, Wong BBM, Norman MD, Stuart-Fox D (2015) Last male sperm precedence in a polygamous squid. Biol J Linn Soc 116:277–287

Thornhill R (1983) Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps.* Am Nat 122: 765–788

Uller T, Olsson M (2008) Multiple paternity in reptiles: patterns and processes. Mol Ecol 17:2566–2580

Voight JR, Feldheim KA (2009) Microsatellite inheritance and multiple paternity in the deep-sea octopus *Graneledone boreopacifica* (Mollusca: Cephalopoda). Invertebr Biol 128:26–30

Wada T, Takegaki T, Mori T, Natsukari Y (2005) Sperm displacement behavior of the cuttlefish *Sepia esculenta* (Cephalopoda: Sepiidae). J Ethol 23:85–92

Wada T, Takegaki T, Mori T, Natsukari Y (2010) Sperm removal, ejaculation and their behavioural interaction in male cuttlefish in response to female mating history. Anim Behav 79:613–619

Wegener BJ, Stuart-Fox D, Norman MD, Wong BBM (2013) Spermatophore consumption in a cephalopod. Biol Lett 9:20130192
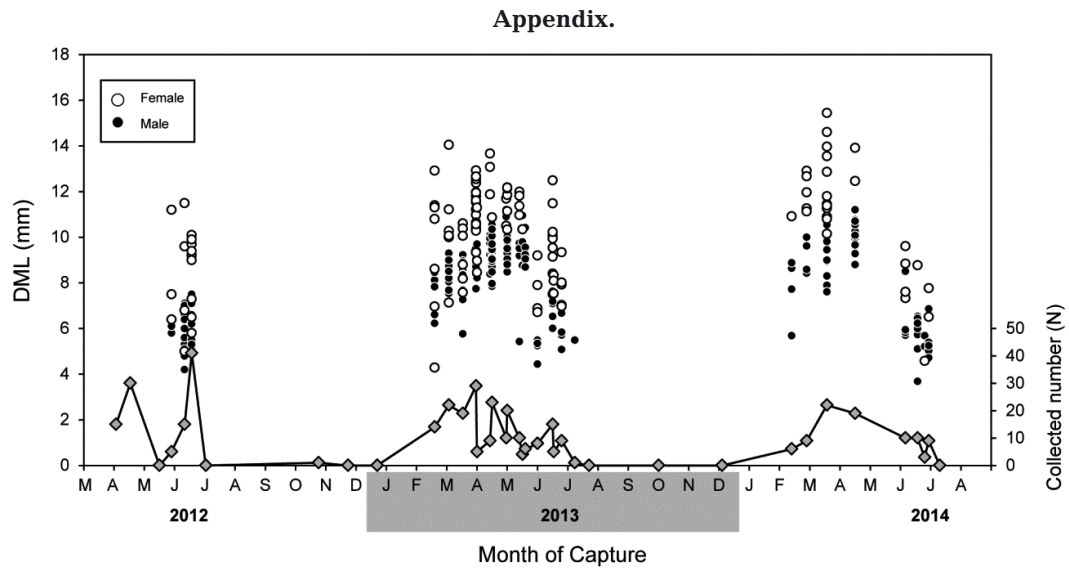
**Appendix.**



Fig. A1. Dorsal mantle length (DML) and number of Japanese pygmy squid collected from 2012 to 2014 in Nagasaki. Grey diamonds: number of collected squid