Doctoral Dissertation

The microsatellite-based demographic analysis of the firefly squid mating system

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March, 2024

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Abstract

Polyandry—the mating style in which a female mate with more than one male—is common in cephalopods. However, I previously found that the firefly squid Watasenia scintillans is mostly monoandrous, based on the observation that only 5% of females have multiple male spermatangia within one individual seminal receptacle (SRs) (Sato, N. et al., 2020). In my doctoral thesis study, I used morphological and genetic analysis to investigate whether the "low polyandry rate" in firefly squid remains low or changes throughout the breeding season. I also examined the relationship between the rarity of polyandrous mating and an overwhelmingly male-biased operating sex ratio (OSR) at the beginning of the breeding season, exploring the mechanism of this peculiar phenomenon.

First of all, females' spermatangium removed from their seminal receptacles (SRs) was subjected to microsatellite analysis to determine their genotype for being male or female. Intriguingly, my data showed that females with 12 or more spermatangia on either one of the two SRs within a female were 100% polyandrous (one female receives sperm from two or more males), whereas females with fewer than seven spermatangia were 100% monoandrous (one female receives sperm from only one male). Among females stored between 8 and 11 spermatangia, the rate of polyandry increased as the number of stored spermatangia increased. This made it possible to determine whether a squid is monoandrous or polyandrous simply by counting the spermatangia numbers of the SR without time-consuming genetic analysis.

Theoretically, male-biased OSR can exacerbate competition among males and increase polyandry. In the present study, we investigated in detail the population change of male and female firefly squids and when females mated (percentage of males who received spermatangia), and under a certain hypothesis (monogamous mating model; Figure 2C in Chapter 2), the OSR at the start and end of the breeding period was estimated to be 32.7 and 9.1, respectively, indicating that firefly squids maintain a highly monoandrous mating system even under highly male-biased conditions.

Next, I calculated the rate of polyandry in a population every week throughout the breeding season using spermatangia number-based probability of polyandry that allows us to estimate a population scale of polyandry. As a result, the polyandrous proportion remained almost constant (a low level between 4 to 11%) throughout the breeding season. Even though the OSR varies greatly through the season, female squids maintain a highly monoandrous mating system during all breeding seasons.

In summary, OSR varied significantly throughout the reproductive period, but polyandry rates remained largely unchanged. These findings imply that the squid mating system is not affected by seasonal variations of OSR. In my doctoral dissertation, I will discuss why rare monoandry mating is adopted in firefly squids and how it is maintained.

List of publications

1 Persistence of a highly monoandrous mating system despite an extremely male-biased operational sex ratio in the firefly squid *Watasenia scintillans.*

Md. Nur E. Alam. & Noritaka Hirohashi

Marine Biology 170, 6 (2023)

DOI: 10.1007/s00227-023-04216-1

2 Context-dependent al plasticity compromises disruptive selection of sperm traits in squid. Noritaka Hirohashi, Noriyosi Sato, Yoko Iwata, Satoshi Tomano, Md. Nur E. Alam, Lígia Haselmann Apostólico, José Eduardo Amoroso Rodriguez Marian

2 **Rare polyandry and common monogamy in the firefly squid, *Watasenia scintillans.*** Noriyosi Sato, Sei-Ichiro Tsuda, Md. Nur E. Alam, Tomohiro Sasanami, Yoko Iwata, Satoshi Kusama, Osamu Inamura, Masa-Aki-Yoshida & Noritaka Hirohashi.

Scientific Reports 10, 1 (2020) 10962.

DOI: 10.1038/s41598-020-68006-1.

Acknowledgments

All glory belongs to Almighty Allah, who has made it possible for me to deliver this dissertation to be awarded a doctorate in philosophy (PhD).

I owe a debt of gratitude and appreciation to my supervisor, Professor Noritaka Hirohashi of the Department of Life Sciences, Faculty of Life and Environmental Sciences, Shimane University, Shimane, Japan, for providing me with the intellectual guidance and support I needed to conduct my research and study. I express my gratitude to him for his invaluable academic guidance, unwavering support, valuable suggestions, constructive criticism and considerate assistance during the research and dissertation preparation.

I would also like to sincerely thank my co-supervisor, Associate Professor Masa-Aki-Yoshida of the Department of Life Sciences, Faculty of Life and Environmental Sciences, Shimane University, Shimane, Japan, for his help and guidance with this research.

Additionally, I would like to express my gratitude to my entire lab team for their cooperative work, kind demeanor and fun work atmosphere.

I would like to thank the International Student Section (I.S.S.) and Faculty of Life and Environmental Sciences officials for their gracious assistance in conducting my study and stay in Japan.

I am eternally grateful to my mother and sisters for their unwavering support, patience and sacrifices, all of which are unpaid. I also want to thank my friends and family for their support and inspiration while I was studying in Japan.

Lastly, I would like to express my gratitude to my wife Nusrat Jahan Nadia and my cherished daughter Sawda Nur Afnan for their support, tolerance, guidance and tenderness.

NUR-E-ALAM, MD

Author declaration

Declaring that this thesis is entirely my own, I, NUR-E-ALAM, MD, undersigned, now affirm. Except in cases where proper credit has been given, as far as I am aware, this thesis does not contain any previously published materials by any other author. The content in this thesis has not been approved for inclusion in any other academic degree program, either in English or another language, or as a requirement for a non-degree program. Entire final revisions are included in this authentic copy of the thesis.

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List of abbreviations

EMP, Estimated Mating Period; **SRs**, Seminal Receptacles;

OSR, Operational Sex Ratio; **SSR**, Simple Sequence Repeat; **TW**, Testis Weight; **ML**, Mantle Length; **SCW**, Spermatophoric Complex Weight; **BW**, Body Weight;

OW, Ovary Weight; **TSI**, Testicular Somatic Index; **OSI**, Ovarian Somatic Index; **ASW**, Average Spermatophoric Weight; **ESS**, Evolutionary Stable Strategy;

PCR, Polymerase Chain Reaction; **gDNA**, Genome DNA;

ROV, Remotely Operated Vehicles.

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Chapter I

1.0. Introduction:

Polyandry, or the mating of a single female with multiple males, is frequently observed in animal taxa and is considered a necessary condition for post-copulatory sexual selection to occur. According to research conducted in the last few decades on behavioral and molecular ecology (Brockmann, H.J. et al.1998; Jones, A. G. & Avise 1997; Griffith, S. C. et al. 2002; Birkhead, T. R. & Pizzari 2002; Parker, G. A. & Birkhead, T. R. 2013). It has been reported that mating with multiple males produces offspring with higher genetic diversity or "good genes" because the females can benefit directly or indirectly from mating with multiple males to a greater extent than they can from mating with a single male (Fisher, D. O. et al. 2006; Boulton, R. A. & Shuker, D. M. 2015). Because there are increased predation risks, disease and virus infections and harassment during courtship or copulation, polyandry is costly for both sexes and it is known that polyandrous females frequently shorten their lifespan (Magnhagen, C.1991; Shuker, D. M. & Day, T. H. 2001; Roberts, K. E. et al. 2015). On the other hand, monogamous animals may have "mutual benefits"(mutual monogamy is a form of relationship between male and female where two partners commit themselves and are sexually involved with one another) for both sexes (Boulton, R. A. & Shuker, D. M. 2015; Laubu, C. et al. 2016; Snekser, J. L. & Itzkowitz, M. 2019) or "unilateral benefit" to one sex over the other. Males often guard females to prevent remating. Females prefer to mate with many males. In this situation, sexual conflict could occur.

Sexual conflict, evolutionary conflicts on reproduction between males and females that occur when a fitness strategy differs between sexes, is one of the major evolutionary theories that covers so many aspects of sexual selection (Komdeur, J. 2001; Hosken, D. J. et al. 2009). Therefore, monogamy is advantageous only in situations in which monopolizing mates is not possible or beneficial. In other cases where monogamy is chosen are biparental care for young and long-term (prolonged) pairing in birds and mammals. These conditions provide a prerequisite for the evolution of monogamy (Hosken, D. J. et al. 2009; Whittenberger, J. F. & Tilson, R. 1980; Young, R. L. et al. 2019). Furthermore, species whose habitats limit their ability to reproduce or to feed, choose monogamous partnerships (Whiteman & Cote, I. M. 2004; Kaiser, S. A. et al. 017). In addition, males (Komers, P. E. & Brotherton, P. N.1997) may become monogamous due to the expense and danger of seeking out additional pairs of partners as a result of female dispersal. However, monogamy remains an evolutionary mystery, as it could have developed in some species without these prerequisites (Kvarnemo, C. 2018).

In cephalopods, in favor of adaptive or alternative outcomes of intrasexual reproductive competition, the majority of coleoid cephalopods exhibit a diverse array of mating behaviors within a short lifespan (usually ranging from 6 months to 2 years) and semelparous reproduction (Iwata, Y.et al. 2011; Squires, Z. E. et al. 2012). Behavioral and anatomical observations have demonstrated that certain coastal species display more than two different reproductive strategies and each related characteristic when engaging in promiscuous mating (Iwata, Y. et al. 2011; Hanlon, R.T. et al. 2002; Hirohashi, N. et al. 2013; Hirohashi, N. et al. 2016; Apostolico, L. H. & Marian, J.2017; Hoving, H.T. et al. 2019). Interestingly, all cephalopod species discovered to date are thought to be polyandrous (Hanlon, R.T. et al. 1999; Franklin, A. M. et al. 2012; Hoving, H. J. et al. 2012; Hanlon, R.T. & Messenger, J. B.2018), presumably due to the lack of environmental (meso- and bathypelagic) barriers to monogamy. However, the diamond squid, *Thysanoteuthis rhombus*, is one species that defies this rule. It may be monogamous (Nigmatullin, C. M. et al. 1995) and appears to establish long-term pair bonds during migration. While genetic analysis is necessary to confirm this phenomenon, additional research is needed to ascertain whether female promiscuity affects the paternity of offspring as well as mating behavior in many other species. It is well known that apparent mating behavior does not always correspond to genetic parentage, regardless of whether it is promiscuous or monogamous (Griffith, S. C. et al. 2002; Lenz, T. L. et al. 2018).

However, it is widely believed that most cephalopods have evolved reproductive strategies and life histories that allow for polyandry; as they are semelparous, have a short lifespan and can store multiple sperm packages for a long time inside the female body (Hanlon, R. T. et al. 2018). Furthermore, to the best of our knowledge, no evidence of biparental care or provisioning by male cephalopods following mating has been reported. If postcopulatory mate guarding can stop both sexes from having the chance to find new partners, it may be a cause of mutual monogamy. Male squid and cuttlefish species frequently exhibit mate-guarding behaviors (Wada, T. et al. 2005; Mather, J. 2016; Naud, M. J. et al. 2016; Allen, J. J. et al. 2017). Nevertheless, it can be temporally or incomplete and extra-pair copulations can occasionally halt it (Mather, J. 2016).

The low population density in the aphotic zones may also contribute to low mate availability, which is another potential reason for monogamy in meso- and bathypelagic cephalopods (Herring, P. J. 2000; Hoving, H. J. et al. 2010; Cuccu, D. et al. 2014). These species mating habits, however, are still unknown. Although there are many unproven situations or circumstances in which monogamy is preferred, the current consensus is that monogamy is unlikely to be a common strategy in cephalopods (Franklin, A. M. et al. 2012; Hoving, H. J. et al. 2012). Most of our understanding of cephalopod reproductive ecology comes from studies with sample sizes, primarily from coastal habitats. This is because behavioral observations can be made in the field or aquariums, or small sample sizes can be used for limited observations such as (Remotely Operated Vehicles) ROVs observations (Lutz, R. A. & Voight, J. R. 1994) or the analysis of cephalopod post-mating signals from collected specimens (Hoving, H. J. et al. 2010; Cuccu, D. et al. 2014). In addition, DNA fingerprinting is a promising method for monitoring mating event outcomes.

I selected *Watasenia scintillans*, the firefly squid, for the present study because its ecological traits are obvious (Hayashi, S.1995), but its mating behavior is still unclear, providing me with the challenge of new insights into cephalopod reproductive systems. Additionally, these squids are valuable commercial resources, enabling fisheries to capture substantial quantities every day during the reproductive season (Arkhipkin, A. I. et al. 2015). The "firefly squid fishery" ban, which is traditionally lifted in Toyama Bay on March 1st due to the particular spawning habitats of this species, is closely related to this. The Sea of Japan, the Sea of Okhotsk and the Japanese Pacific coast are the places where firefly squid is most commonly found. Over 200 meters below the sea surface during the day, adult squids can be found there and at night, they migrate up to 50 to 100 meters below the surface (Hayashi, S.1995). In the spawning season, females may spawn thousands of eggs at a time and possibly even multiple times, whereas males do not appear and therefore do not participate in this activity. Females store sperm within male-derived spermatangia that are attached to the seminal receptacle situated under the collar on the bilateral sides of the nuchal cartilage (Hayashi, S.1989). In order to confirm my recent finding that *W. scintillans* have monogamous mating (Sato, N. et al. 2020), I repeated key experiments in line with this report. In this chapter, I provide genetic, morphometric and demographic evidence for this trend.

1.1. Materials and Methods

1.1.1. Sample collection

The firefly squid, known as “Hotaru Ika” in Japanese was obtained through fishery catches using bottom trawls towed around Sakai-port in Tottori Prefecture and Oki Islands in Shimane Prefecture in Japan, using fixed nets set up around and close to the shelf break in the innermost section of Toyama Bay in Toyama Prefecture, Japan. The commercial fishing season for this species runs from March to May in Toyama and from January to May in Shimane/Tottori. Specimens collected in Shimane/Tottori from 2016 to 2020 were used for all morphometric measurements.

1.1.2. Measurement of the squid specimens:

The measurements of the squid specimens included testis weight (TW), spermatophoric complex weight (SCW), dorsal mantle length (ML), total body weight (BW), number of spermatophores stored in the sac and terminal organ, ovary weight (OW) and number of spermatangia on the left and right sides (left, 6.23 ± 1.58; right, 6.31 ± 1.57; n=1,930) of the female seminal receptacle. These measurements were taken mostly within a day of the fishing expedition. The number of spermatangia was counted using a stereomicroscope. As $TSI=100 \times TW \times BW^{-1}$ and $OSI=100 \times OW \times BW^{-1}$, respectively, the testicular somatic index (TSI) and ovarian somatic index (OSI) were computed. After fixing the spermatophoric complex in 10% formalin in seawater, it was dissected under a stereoscopic microscope to determine the number of spermatophores. All males of the *W. scintillans* species were either mature or spent (69 classified them as stage V or VI). Information for the other squid species was obtained from the literature. SpN, or the number of spermatophores stored in the spermatophoric sac, is indicated by the formula $100 \times (SpN \times ASW) \times (TW+SCW)^{-1}$, which is used to estimate the average spermatophore weight (ASW) in each individual. These calculations were based on the regression lines shown in Figures 2B and C. 100× TW× (TW + SCW) −1 was used to allocate resources to the testes.

1.2. Results

1.2.1. Seasonal dynamics of population and reproduction in *W. scintillans*

In the population of *W. scintillans* caught by local trawl fishing near the Oki Islands in the Sea of Japan, I examined seasonal variations in sex ratios and the prevalence of female virginity (i.e., the females that have not mated yet). During spring, between mid-February and mid-March (hereafter referred to as the estimated mating period or EMP), I observed a rapid disappearance of males and virgin females. Notably, implanted female spermatangia were kept on bilateral sides of the seminal receptacle (Fig. 1A), with approximately six spermatangia on each side. Throughout the reproductive season, this pattern remained constant, with the number of spermatangia gradually decreasing. According to Sato, N. et al. (2020), the number of days needed to lose one spermatangium from the left or right side, as estimated from a regression line, was 175.4 or 192.3, respectively. And my results agreed with this finding. This suggests that spermatangia are preserved for the duration of their life once they are attached to the female's seminal receptacle.

1.2.2. Estimation of male mating opportunities

The maturity and fecundity of *W. scintillans* individuals were measured quantitatively and morphometrically before, during and following the EMP. It was obvious that stored spermatophore numbers in the Needham's sac, their storage organ, increased during the breeding season, except for the EMP (Fig. 2), when males utilize their spermatophores. Males do not copulate after EMP, based on the possibility that a similar continuous increase in the number of stored spermatophores is observed in the periods preceding and following EMP. As the mean number of spermatophores received by females was 12, the mean number of male-stored spermatophores immediately before EMP (pre-EMP) was approximately 30, indicating that males could copulate no more than two or three times. In the pre-EMP stage, females have just started to mature and will need a few more weeks to become fecund (Fig. 3 A), while males reach full maturity with the highest testicular-somatic index (TSI), (Fig. 4), a measure of sperm-producing capacity or promiscuity. Thus, I speculate that males of *W. scintillans* may have chosen a monogamous mating pattern because of their low capacity to produce sperm, the limited number of mating opportunities resulting from a male-biased operational sex ratio and the lack of female remating attempts.

1.3.0 Discussion

In the spring, female firefly squids migrate to shallow water to spawn, but males leave the coastal zones far sooner than females (Arkhipkin, A. I. et al. 2015). This indicates that males live one month less on average than females do (Hayashi, S 1995; Yuuki, Y. et al. 1985). This scenario was supported by the coincidental disappearance of virgin females and males (Fig. 5). To preserve spermatozoa internally before spawning, females must do so for a considerable amount of time. Males use their hectocotylus to deposit sperm packages or spermatophores on or inside females during copulation, which is characteristic of most coleoid cephalopod reproduction. Following the deposition of spermatophores, a complicated evagination process known as the spermatophoric reaction occurs, leading to the stable implantation of the ejaculatory apparatus (spermatangium) into female tissue (Hanlon, R.T & Messenger, J. B 2018; Marian, J. E. A. R. 2015). Since promiscuous mating results in females receiving spermatangia from several males concurrently or successively, mixed populations of spermatangia are dispersed erratically around the female's deposition sites. On the other hand, I have consistently observed that the females of *W. scintillans* retain masses of spermatangia in precisely the same locations on both sides of the nuchal cartilage, about six on each side. It is unordinary for cephalopods to have such a profoundly standard design of spermatangium arrangement (Burgess, L. A. A. 1998). This made me wonder if all of these spermatangia came from a single male. In most of the coleoid cephalopods during their reproductive season, males deposit their sperm on the female’s seminal receptacle under the collar of bilateral sides of nuchal cartilage. The number of spermatangia of the females comes from a single male in most cases or various males gradually or at a time. However, the females *W. scintillans* were found to have approximately 6 spermatangia on each side of the seminal receptacle (Fig. 1 A), which was continued until the end of the fishery season. This suggests that the spermatangia were transferred only once in *W. scintillans* and thereafter remained attached to the female.

The paternity analysis of each spermatangium comes from a single female and completely revealed that the greater portion of female squid *W. scintillans* mate with only one male partner during their reproductive season. DNA analysis also reported that no DNA mismatch between spermatangia and embryos that came from the same female and proved that females are monogamous（Sato, N. et al. 2020 suppl Figure 1）. The adult sex ratio is largely male-biased before and during the EMP (Fromhage, L. et al. 2005). I found that the majority of *W. scintillans* female`s mate with only one male, indicating behavioral monandry, based on genetic data (the paternity of each spermatangium stored in any one female). Because of one exceptional instance in which a female mated with three males, our results, however, need to be interpreted with caution. It is plausible that the global range of habitat (from the East China Sea to the Okhotsk Sea) would allow firefly squids to exhibit adaptability in selecting their mating pattern, given that female promiscuity can be adaptive in response to extrinsic conditions like an increase in predation risk (Hedrick, A. V. & Dill, L. M.1993).

Females can spawn eggs multiple times at specific intervals during the reproductive season (Hayashi, S. 1995). However, because males disappear before females are fully fertile (Fig. 5) and because the spontaneous loss of once-attached spermatangia to females rarely occurs other males are unable to engage in replenishing the spermatangia during these intervals. Male-biased sex ratios and infrequent female reproduction thus restrict male mating opportunities. Consequently, at the start of the EMP and continuing through it, the tertiary (adult) sex ratios and the operational sex ratios (Fromhage, L. et al. 2005) were significantly skewed toward males (Fig. 5, See also Fig. 2 in Chapter 2). A fixed evolutionary stable strategy (ESS) for males mating with only one partner is predicted by mathematical modeling under these circumstances (Fromhage, L. et al. 2005; Gomes, B. V. et al. 2018). I conjecture that in this species, male monogyny gave rise to mutual monogamy (Fromhage, L. et al. 2005), after which female monandry was established. If male monogyny becomes an ESS, such evolution might reward males who focus more of their energy on activities other than sperm production, or fecundity (Simmons, L. W & Garcia-Gonzalez, L. 2008; Baker, J. et al. 2020). This explains why this species invests little in spermatangia, or the opportunity for copulation and testicular function, or the production of sperm. Males could have adapted to a mechanism of first-male sperm precedence (Bonduriansky, R et al. 2008) by using a "live-fast-die-young" life history strategy, rather than directing their reproductive investment toward greater fecundity (Fig. 5). We hypothesize that female-driven monoandry (Simmons, L. W. & Garcia-Gonzalez, L. et al. 2008; Baker, J. et al. 2020) is what led to this low level of male fecundity.

It is clear that certain elements that have been linked to monogamy in other taxa like long-term pair bonding, biparental care, low population density, habitat restriction, time limits for reproduction and enforcement (mate guarding)—do not apply to this species (Wittenberger, J. F. & Tilson, R.L. et al. 1980; Whiteman, E, A & Cote, I. M. 2004; Kvarnemo, C. 2018; Reichard, U. H. 2003). The reason for this is that, before the main spawning season peaks, the males go completely missing and they make shortened spawning migration terms. Alternatively, because this species is the dominant prey for demersal fishes (Yamamura, O. & Inada, T. 2001) and is subject to high levels of predation, lengthy courtship behavior may be detrimental to the species' survival, which would lower the success of multiple mating for both sexes (Magnhagen, C.1991; Rodriguez- Munoz, R. et al. 2001; Franklin, A. M. et al. 2014). Predation risk is predicted to act as an evolutionary force favoring monandry and some examples support this theory (Magnhagen, C. 1991). Male-specific courtship signaling is generally not as attractive to non-virgin (mated) females across taxa and the underlying mechanisms are not always the same because of physiological, physical and behavioral changes that occur after mating (Wedell, N. 2005; Guevara-Fiore, P. et al. 2009; Ruthera, J. et al. 2010). Nonetheless, the effects of these female adaptation seem primarily to be protective against predators. Any species that can successfully reproduce should be able to adapt to a dark environment, even though the actual mating habits of deep-sea organisms are mainly obscure or unknown (Herring, P. J. 2000). The Octopodiformes squid *Taningia danae* is thought to engage in communication through bioluminescence signaling, which could be interpreted as courtship behavior (Kubodera, T. 2007). The ability to differentiate between conspecific illumination (green) and environmental down-welling light (blue) (Seidou, M. et al. 1990) may be attributed to *W. scintillans* special eyes, or photoreceptor cells, which contain three visual pigments with varying maximum wavelengths (~471, ~484, and ~500 nm). Therefore, the firefly squid's bioluminescence may contribute to once-in-a-lifetime mate search or courtship signaling.

In the first part of the doctoral thesis, I presented the reproductive characteristics of this squid species through a fine-scale analysis of seasonal dynamics in each individual's demographics, mating status, and reproductive indices from fishery catches (Md. Nur E Alam & Noritaka Hirohashi. 2023). In the second part of the thesis, I will explain further genetic evidence for a major monandrous mating pattern and detailed seasonal change of polyandry level in firefly squid.

Figures

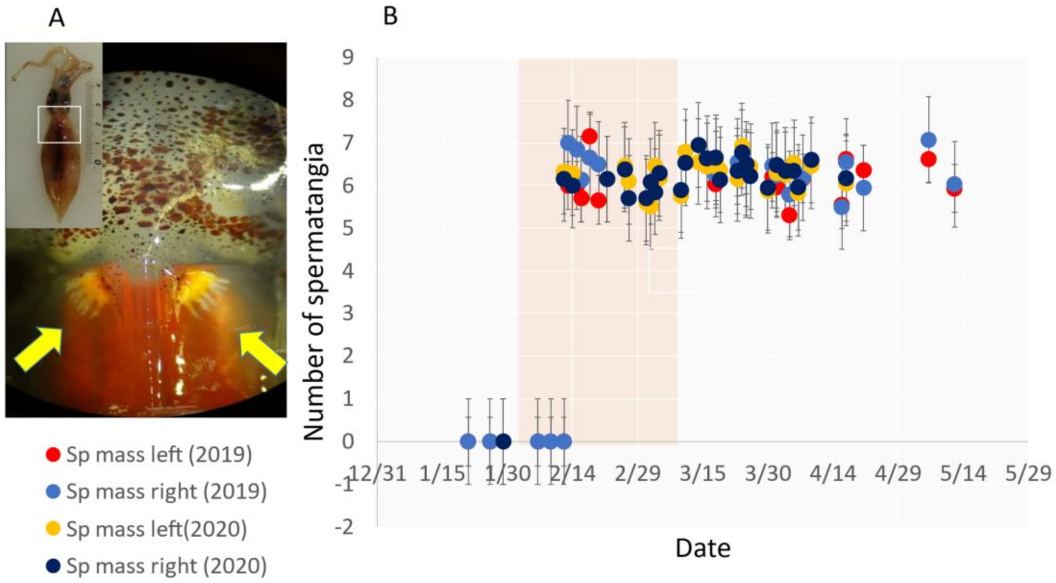

Figure 1: Permanent and stable storage of the spermatangium (a sperm-storing apparatus) in females

A, a picture shows the female seminal receptacle (*arrows*) where the spermatangia were attached. Inset is an image of a female`s whole body. B, the number of spermatangium attached on the bilateral sides of the female seminal receptacle was counted. At the beginning of the season, none of the females stored the spermatangia. Immediately after entering the expected mating period, females were found to carry approximately six spermatangia on each side of the seminal receptacle, which was continued until the end of the fishery season. This suggests that the spermatangia were transferred only once and thereafter remained attached to females.

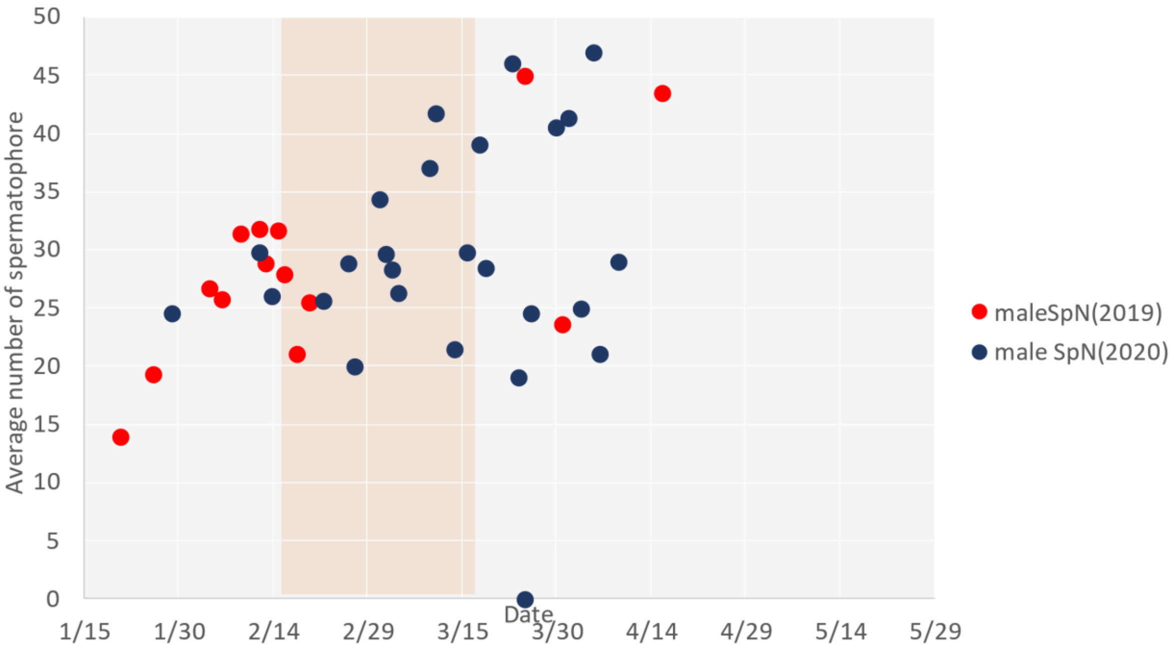

Figure 2: The dynamic equilibrium of male-stored spermatophores (sperm packages) between their production and consumption (copulations)

Males accumulate spermatophores in their spermatophoric sac (the storage organ) during the early season and reach a maximum by the onset of the expected mating period. First week of the expected mating period, the number of stored spermatophores was constant or slightly decreased, perhaps due to the equilibrium between their production and usage. After the expected mating period, the male-storing spermatophores were again increased, suggesting that no more copulation opportunities for males.

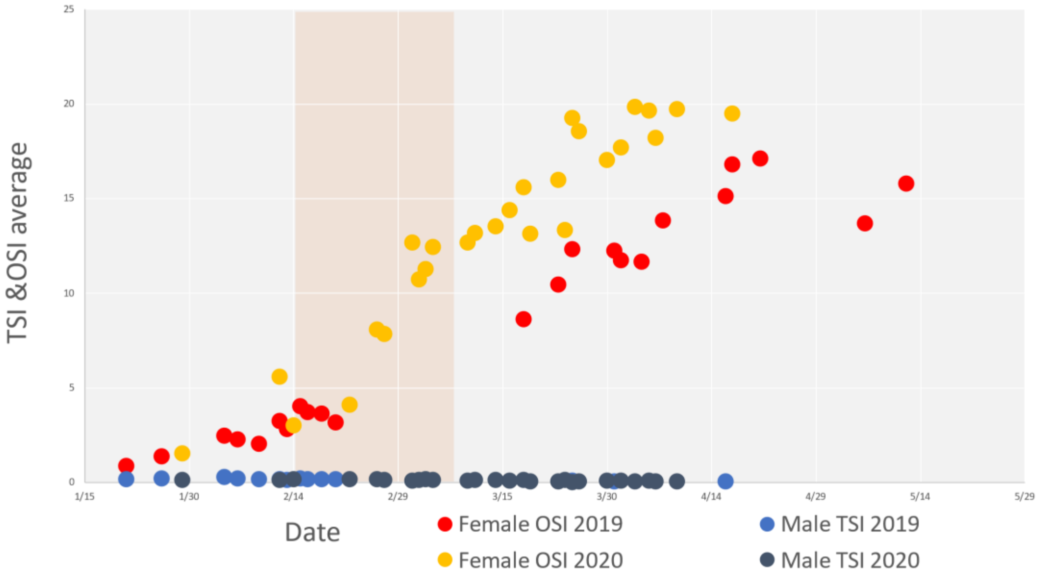

Figure 3: Males become mature before a substantial increase in ovarian size

Throughout the season, ovarian somatic index (OSI), a measure of female maturity or fecundity showed a gradual increase. These data agree with the sex difference in the life history of this species; males die soon after copulation and females continue growing and then spawn eggs thereafter.

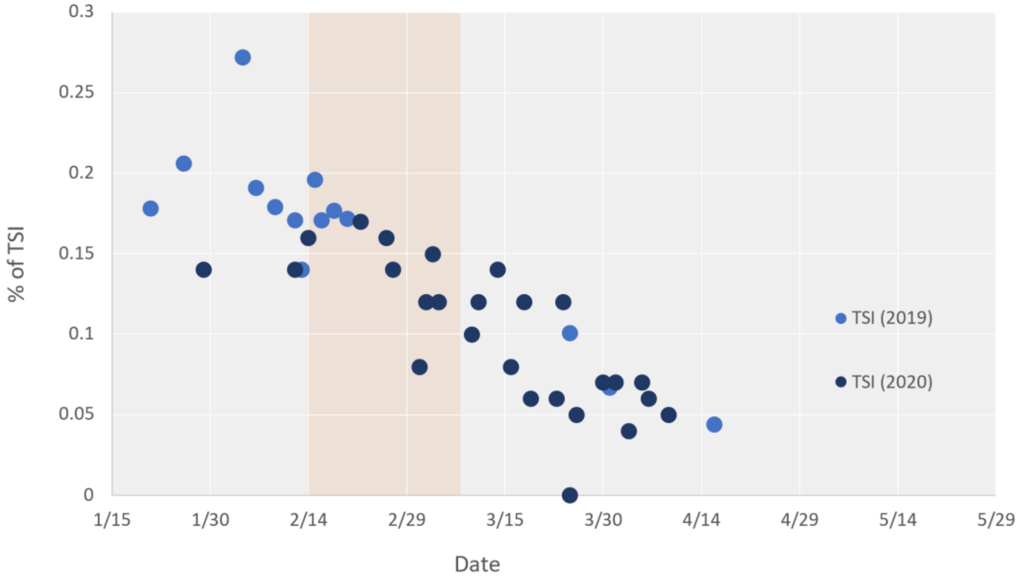

Figure 4

Testicular somatic index (TSI), often accounted for male maturity or promiscuity. Early in the season of firefly squids, I observed that males were matured before the females. TSI reached its highest peak just before the expected mating period.

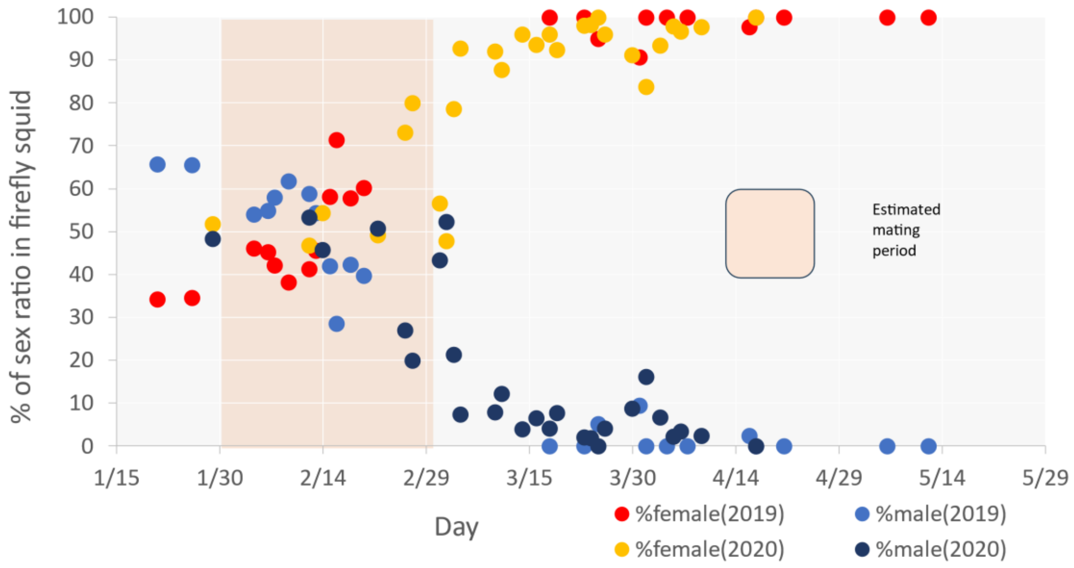

Figure 5: The seasonal changes of adult sex ratio (ASR) in the firefly squid

In early the season (before the expected mating period), ASR is slightly biased toward males (% male > 60), followed by a gradual decrease in the male population during the expected mating period. By the end of the expected mating period, almost all males disappeared, presumably because of male's lifespan was much shorter than the female's (Sato, N. et al. 2020).

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Chapter II

2.0. Introduction:

One of the main mechanisms by which selection can take place is mate choice. It is distinguished by a "selective response by animals to particular stimuli” (Emlen, S. T. and Oring, L. W. et al. 1977), which can be seen in behavior. In other words, before an animal interacts with a potential mate, they first assess various qualities of that mate, such as the resources or phenotypes they possess and assess whether or not that particular trait is beneficial to them. The evaluation will then receive a reaction of some kind. Evolutionary biologists pay close attention to the specific times when females select males to be their partners for mating, a process known as female choice (Emlen, S. T. & Oring, L. W. 1977; Eberhard, W. G.1996; Rosenthal, R. R & Ryan, M. J. 2022).

The complex behavior, known as agonistic behavior, is typically displayed by males, including fighting, threat, appeasement and avoidance, courtship. The agonistic behavior involves coordinating the activities of sexual partners in time and space and increases sexual motivation, mating, but does not typically involve fertilization in cephalopods. And any type of parental care, if it occurs, are all examples of reproductive behavior (Hanlon, R.T & Messenger, J. B. 2018). Reproductive strategies, which cover a wider range of relating to the time of reproduction and the trade-off between quantity and quality of offspring, overlap with mating strategies. However, it is still largely unknown how females choose how many males to mate with, especially when there are only one or a few males (Jennions, M. D. & Petrie, M. 2000; Kvarnemo, C. 2018).

The fact that males exhibit more competition for mates compared to females shows that the discrepancy in selection intensity between sexes is the result of rivalry with in the male population. The number of mates is determined by courtship, which also plays a role in intra-sexual selection (Bateman, A. J.1948). I emphasize that polyandry is still variable even though I agree that is a generally widespread phenomenon. Because males produce a huge amount of sperm and may fertilize all of their partner's eggs, male fitness is positively correlated with the number of matings in many animals (Bateman, A. J.1948). The upper limit of fitness for females is determined by their limited egg production, whereas male fitness increases monotonically in response to multiple mating (Bateman, A. J. 1948). The evolution of female multiple mating, which is observed in a variety of animal and plant taxa, may be explained by bet-hedging via polyandry - spreading the extinction risk of the female’s lineage over multiple males (Matsumura, K. et al. 2021).

It is commonly believed that the way animals reproduce, whether it's with just one partner or with multiple partners. This is seen as a fixed characteristic for each species. (Whiteman E, A. & Cote, I. M. 2004; Hughes, W. O. et al. 2008; Davies, N. G. & Gardner, A. 2018; Young, R. L. et al. 2019). Regrettably, monoandry (or monogamy) is presumed order to achieve the maximum to have already been implemented as a necessary consequence of biparental care and severe constrictions on the accessibility and availability of reproductive performance or environmental systems in one or both sexes (Tumulty, J. et al. 2014; Komers, P. E. & Brotherton, P. N. 1997; Stockley, P. 1997).

As a necessary consequence, it is increasingly acknowledged that the choice of reproductive mode is largely dependent upon environmental, intrasexual, or intersexual contexts and differs significantly between populations (Uller, T. and Olsson, M. 2008; Brown, J. L. et al. 2010). According to Arnqvist, G. and Nilsson, T. (2000); Kokko, H. and Jennions, M. D. (2008); Janicke, T. & Morrow, E. H. (2018) and others, the operational sex ratio (OSR), or the proportion of males to females who are ready to mate, may have an impact on the choice of reproductive mode in a given population. (Head, M. L. et al. 2008; Plesnar-Bielak, A. et al. 2020).

If the OSR is biased toward one sex, mating competition becomes more intense (Trivers, R. L.1972; Klug, H. et al. 2010). This leads to altered mating behaviors, such as an increase or decrease in competitive aggression, courtship, pre- or post-copulatory mate guard and frequency or duration of copulation (Kvarnemo, C. & Ahnesjo, I. 1996; Weir, L. K. et al. 2011). I assumed that in species lacking in aggression, courtship, mate guarding and parental care, a possible all change that may occur in response to increased male-biased OSR is a higher frequency of copulation with higher levels of polyandry (Lode, T. et al. 2004; Naud, M. J. et al. 2004). The firefly squid *W. scintillans* provides a model system that is appropriate for testing this hypothesis because (1) it is semelparous and unlikely to engage in courtship and mate guarding due to a significant amount of time passing between copulation and spawning (Hayashi, S.1995). (2) Since females make up about 5% of the polyandrous population (Sato, N. et al. 2020), I can detect a slight increase in polyandry; (3) During the mating season in the wild, the adult sex ratio significantly shifts from being male-biased to female-biased (Sato, N. et al. 2020); (4) A newly created anatomical method, which is described in the current study, can be used to estimate the degree of polyandry at a large population scale. I am intrigued by the highly monoandrous reproductive mode in *W. scintillans* in addition to this query regarding the potential impact of OSR on decisions regarding monoandry versus polyandry as suggested by field observations and paternity tests using DNA fingerprinting (Hanlon, R.T. et al. 1997; Naud, M. J. et al. 2004, 2016; Iwata, Y. et al. 2005; Sato, N. et al. 2014) Males are in the wild matured before females and then wait (Sato, N. et al. 2020). How often female animals are mating? Most female animals mate only once. Early sexual stage during the brief mating time of an animal. Ants (Arthropoda) keep male-delivered sperm sacs for up to 3 weeks and store them for no more than 3 months. (Sato, N. et al. 2020).

Most females copulate only once at the sexually premature stage during the brief mating period of three weeks. Behavioral monogamy (all sperm sacs stored in the female were transferred by a single male), was shown by microsatellite DNA-based paternity analysis. As a result, genetic monoandry (all eggs in the same clutch, despite investigation with small sample size, were fathered by a single male) was observed (Sato, N. et al. 2020). Inferring two possibilities from these data is what I did: If certain conditions are met, polyandry can either occur as an alternative common strategy or as a result of unusual coincidences or pathological behavior (options 1 and 2). The difficulty of answering these questions is hampered by the fact that microsatellite DNA analysis necessitates a significant amount of work and thus restricts large-quantity analyses, such as population dynamics. To find out if a population's rate of polyandry can alter in response to seasonal dynamics of the OSR, however, it would be of great interest. As the mating season progresses in firefly squid, the adult sex ratio dramatically shifts from male-biased to female-biased (Sato, N. et al. 2020). According to our observation, the OSR is heavily biased toward males at the start of the mating season because males are fully mature while females are only beginning to reach sexual maturity (Sato, N. et al. 2020) and after that, due to the massive disappearance of males (likely caused by early death), it becomes strongly biased toward female. As a result, I assume that male-male competition for mates would be significantly greater early in the mating season than later in the season. The rate of polyandry should be significantly higher in the early season if copulation is predominately controlled by males, i.e. if female mate choice is ineffectively managed.

2.1.0. Materials and Methods

Between 2015 and 2022, the firefly squid species were purchased from nearby fisheries during the fishing season (Jan.-May). The squids are typically caught by bottom trawls that are towed close to the Oki Islands (Shimane Prefecture, Japan) and Sakaiminato-port of (Tottori Prefecture, Japan) and they are then transported as dead specimens in ice-cold containers to the markets in the day of fishing. Before use, the fresh samples were stored frozen (- 20°C). A total of 51 sampling days from the data collected in 2019 and 2020 were used for the analysis of OSR and at least 50 individuals were examined for the fundamental measurements (sex, mantle length, body weight, testis mass, gonadosomatic index and ovary weight) on each day. Based on the formula 100 x (ovary weight) x (body weight), relative ovary weight (OSI) was determined.

2.1.1. Genotyping of female-storing spermatangia using microsatellite DNA markers

The process of genotyping was completed as previously described (Sato, N. et al. 2020). First, I counted the spermatangia on each seminal receptacle and I categorized the females based on these counts. I always examined the genotypes of females because it is uncommon for them to have more than 10 spermatangia. Genotyping was always done using spermatangia that had been taken from just one site of a female's nuchal pocket (by selecting the pocket with the highest number of spermatangia). I chose at least 20 females at random among those with 7-9 spermatangia per site. Males who had successfully mated had their spermatangia removed from their seminal ovary. With the aid of fine forceps, they were divided into each spermatangium and put into a Petri dish that was 70% filled with ethanol. Each storage location's quantity of spermatangium was counted. Each spermatangium was lysed for 4 hours at 52 °C in 50 µl of 50 µg/ml Proteinase K-containing CTAB (100 mM Tris-Cl pH 8.0, 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid, 2% cetyltrimethylammonium bromide) with continuous stirring and then centrifuged for 10 minutes at 4 °C. After the supernatant was transferred to a new tube, the usual phenol/chloroform protocol for extracting genomic DNA was used. bromide) in a 1.5 ml test tube with constant stirring for 4 hours at 52 °C, then centrifuged for 10 minutes at 4 °C at 14,000 rpm. After transferring the supernatant to a new tube, the genomic DNA was extracted using the standard phenol/chloroform method. After being precipitated with 0.3 M sodium acetate (pH 5.2) and 70% ethanol, the genomic DNA (gDNA) was air-dried, washed with 70% ethanol and then dissolved in 40 ml milli-Q water. To quantify gDNA, 0.8% agarose gel electrophoresis was carried out. With some modifications from what was previously described (Sato, N et al. 2020), microsatellite markers were used for genotyping. Briefly, 0.2 µM primers with FAM, Hex, Cy3 and PET-tagged sense oligonucleotides and 100–300 ng gDNA were used in a polymerase chain reaction (PCR) with the KAPA2G Robust PCR Kit from NIPPON Genetics. The PCR conditions were 95 °C for three minutes, followed by 30 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds, 72 °C for 15 seconds and 72 °C for five minutes. Using the ABI PRISM 3130xl genetic analyzer and GeneScanTM600 LIZ dye size standards, the fragment length analysis was carried out. To analyze the discovered peaks, we used OSIRIS-2.15.1 (National Institute of Health, USA).

2.1.2. Estimation of operational sex ratio:

In the beginning, OSR was determined as the proportion of mature males to females in a population who are ready to mate. I considered all male individuals to be mature and always ready to mate throughout the mating period because they all had spermatophores in their storage organs for at least a week before the start of the mating period until they completely disappeared. Females, on the other hand, had underdeveloped ovaries and over the course of the following three weeks, the proportion of virgin females gradually decreased to zero. I viewed the virgin females as "not being ready to mate" (Fig. 2C) because mature males and females with or without stored spermatangia cohabitate during this period (as if they were caught in the same net). As a result, a daily rise in the proportion of non-virgin females indicates a significant rise in the number of females who have become ovulatory within the past 24 hours. OSR was empirically determined by counting the number of males and females who were ready to mate (Kvarnemo, C. & Ahnesjo, I. 1996), taking into account two distinct scenarios for the female mating regime: both monoandry and polyandry according to the polyandry regime, a female's reproductive status changed from "not being ready to mate" to "being ready to mate" (Fig. 2C) at a specific point during the reproductive season. As a result of the monoandry regime, where the reproductive status of "being ready to mate" was thought to have ended with the first mating, the status of "not being ready to mate" was not reached until the end of the reproductive season. Because they failed to mate despite being with mature males, the virgin females collected during the estimated mating period (February 4-March 4) were considered to be "not ready to mate." (Fig. 2C). Given these circumstances, the following formula was used to calculate the temporal (daily) OSRs during the mating season:

Polyandry regimented OSR = the mean number of males collected today ⁄ the mean number of non-virgin females collected today

Monoandry regimented OSR = the mean number of males collected today ⁄ (the mean number of non-virgin females collected today− the mean number of non-virgin females collected yesterday.

The rate of change from "not being ready to mate" to "being ready to mate" (Fig. 2C) is represented by the daily increase in the proportion of non-virgin females. This transition was fitted to a linear regression equation, which allowed us to calculate the average transition rate for the female population as 3.73%/day. In addition, I was able to streamline the OSR calculations due to the linear decline in the male population during the mating season. I calculated the OSRs using linear regression equations.

2.2.0 Results

2.2.1. A correlation between the rate of polyandry and the number of spermatangia attached to female

The majority of monoandrous females had an average of 6 spermatangia (Part-I fig. 1) at each SR (left, 6.23 ± 1.58; right, 6.31 ± 1.57; n=1,930), whereas one exceptional female with unusually high numbers of spermatangia (12 on the left and 13 on the right) was polyandrous (Sato, N. et al. 2020). As a result, I genotyped each spermatangium in a pair of SRs from 141 females, splitting them in half. In contrast, females with 7 spermatangia were only monandrous. I discovered that females with 12 spermatangia on either of the two SRs within a female were all polyandrous (Fig. 1A). In females storing between 8 and 11 spermatangia, the rate of polyandry increased as the number of spermatangia increased (Fig. 1A) of attached spermatangia on the female SRs might affect female multiple mating. The number of sires increases with increasing spermatangium number in females with 8 or more spermatangia in the SR (Fig. 1B). These findings imply that one can estimate the likelihood of polyandrous mating based on the number of attached spermatangia.

2.2.2. Estimation of operational sex ratio during the mating period:

I calculated the rate of increase in mated females in a population and discovered that the rate of increase can be roughly predicted by a linear regression model (Linear regression, $r^2=0.857$, $F_{1,19}=102.13$, $P<0.0001$) when the mating period is narrowed by trimming the ambiguous starting and ending points of the mating period (Fig. 2A; dashed red box). This results in a constant increase of 3.73% per day. Additionally, I could calculate the sex ratio at any given time using information gathered over the previous three years at the same fishing site. This revealed a linear decline (Linear regression, $r^2=0.831$, $F_{1,35}=162.62$, $P<0.0001$) in the male population (1.18%/day) during the mating season (Fig. 2B; dashed red box). These estimates, along with the polyandry/monoandry mating regimes, were taken into consideration when calculating the OSR and the results showed an exponential decline in the male-biased OSR over time (Fig. 2D; purple plots). Notably, the OSR changes from being biased toward males to being biased against females in the middle of the mating season due to a sharp decline in the population of males. After that, I looked at their strict monoandrous mating system and tested an alternative model in which females do not engage in remating activities after having copulation with the first males, so they are regarded as having lost their receptivity to the second male's copulation and becoming "not ready to mate" (Fig. 2C; green box). In this scenario, the male-biased OSR decreased significantly (during the mating period, from 32.7 to 9.1) but remained essentially at high levels (Fig. 2D; green plots).

2.2.3. Estimation of the rate of polyandry during the reproductive season

The probability of polyandry related to the spermatangium number (integrated probability of polyandry) was estimated and used the formulate the equation based on these findings. Using a total of 5,303 females obtained between 2015 and 2022, I estimated the percentage of polyandry in squids who were caught during the same week of the year using the following equation：

$$\text{Integrated probability of polyandry } (P_{int}) = \Sigma x p_{i,j} \ / \ \Sigma x_{i,j} \qquad 1)$$

where $p_{i,j}$ indicates the probability of polyandry of females with i (number) of spermatangia on one site and j (number) of spermatangia on another site. And $\Sigma x_{i,j}$ indicates the total number of females examined. Every p values were calculated from experimental data shown in Fig. 1A.

$p_{8,\leq 8} = 0.1$; $p_{9,\leq 8} = 0.25$; $p_{10,\leq 8} = 0.39$; $p_{11,\leq 8} = 0.79$; $p_{9,9} = 0.25$; $p_{9,10} = 0.39$, $p_{9,11} = 0.78$; $p_{10,10} = 0.39$; $p_{10,11} = 0.78$; $p_{11,11} = 0.79$; $p_{\geqq 12,any} = 1.0$

Throughout the reproductive season (February to May), the rate of polyandry fluctuated on a weekly average between 4.06 and 11.72% without showing any clear trends of changing patterns. It was calculated that 8.03±2.63% of this species' population was polyandrous.

2.3.0 Discussion

It is well known that females frequently mate with multiple males (polyandry) in sexually reproducing organisms. With very few exceptions, polyandry is also common in cephalopods (Nigmatullin, C. M. et al. 1995; Sato, N. et al. 2020; Murai. R. et al. 2021). A notable exception is the deep-sea squid *W. scintillans* (Sato, N. et al. 2020). I previously estimated that only 5% of this species exhibits polyandry based on analyses of microsatellite DNAs and extensive, season-wide anatomical investigations (Sato, N. et al. 2020). The genetic evolutionary changes happen in monoandrous reproductive mode whereas most of the cephalopods represent polyandrous. The time constraints for available mate search may be a potential factor affecting remating frequency and motivation because the mating period is thought to be as brief as 3–4 weeks (in February) and males disappear from the fishery grounds after this period.

Male`s low fertility may also discourage multiple copulations, as evidenced by the fact that their relative testis weight, a proxy for male promiscuity, is incredibly low (Sato, N. et al., 2020) and that each male stores only about 30 spermatophores on average. Additionally, females store male-delivered spermatangia with an average of six at each pair of seminal receptacles (about 12 spermatophores/female) and can store sperm for an extended period until the end of the reproductive season. All of these circumstances are likely to favor a monogamous mating strategy. However, the evolutionary process by which monogamy has been adopted in this species is still unknown. According to some theories (Manning, A. 1962; Wedell, N. 2005;

Guevara-Fiore, P. et al. 2009; Ruther, J. et al. 2010; Xochipiltecatl, D. et al. 2021), monogamy develops when an individual's intrinsic mechanisms and environmental factors prevent them from seeking out a second mate. In the case of firefly squid, mature males are prepared for mating and cohabiting with females, suggesting that mating choices primarily depend on females' receptivity to mate. In that case, I assumed that the operational sex ratio (OSR) might be biased in favor of males at the start of the mating season.

According to some reports, a single sex-biased OSR may have an impact on a variety of male mating behaviors, including how frequently males mate (Pitnick, S. 1993; Markow, T. A. 2002). As a result, I made an effort to determine the exact values of OSR in two distinct scenarios: the "polyandrous mating regime," in which females maintain their receptive status after mating and the "monoandrous mating regime," in which females lose their receptivity to mate after copulating with a first male. According to each scenario, I calculated the OSR and the results show that the OSR is significantly more biased toward males at the beginning of the mating period (in the polyandrous mating regime) or throughout the mating period (in the monoandrous mating regime) (Fig. 2D). Based on our earlier discovery that 95% of females were monoandrous at mating (Sato, N. et al. 2020), I believed the second scenario of a "monoandrous mating regime" to be appropriate for this species.

To precisely estimate population-scale polyandry and its seasonal variations, I adopted a novel methodology to answer this question. In the past, I used four microsatellite loci to genotype 272 spermatangia from 19 females (Sato, N. et al.

2020). Nevertheless, figuring out the population dynamics of polyandry proved to be challenging with this method. Because of this, I integrated morphological analysis and genotyping, drawing from our recent discovery that the likelihood of multiple mating is correlated with the number of attached spermatangia (Fig. 1A). My ability to apply this new method to the mathematical analysis for polyandry estimation has enabled us to include a large sample size that has been gathered over the previous eight years. In my earlier report, during the reproductive season, there was a very low incidence of polyandry. According to statistics based on weekly averages, the rate of polyandry varies from 4.06 to 11.72% (Fig. 3), exhibiting inconsistent patterns that change over time. I believe that the exact location of fishing points within a fishing field may have an impact on the rate of polyandry because I have occasionally observed variations in the sex ratios within different fishery transports (fishing points) on the same day. However, despite a notable shift in OSR, our data currently indicate that females continue to use a monoandrous mating system.

According to a paradoxical theory, females would experience an excessive number of copulation opportunities due to their tolerance of copulations following concurrently changing OSR in the mating field. This would result in an excessive amount of risk associated with copulation, including predation, infection and injury (Marian, J. E. A.R. 2012). Therefore, I assume that virgin females are prevented from remating in favor of a cost-benefit trade-off under highly male-biased OSR conditions. Given this, a fascinating question about the evolution and maintenance of squid mating is raised by the unusual occurrence of polyandrous mating in this species.

This study aimed to estimate the dynamics of the OSR in a time-resolved manner by dividing the mating regimes into two categories: "time-in" and "time-out," respectively (Clutton-Brock, T. H. & Parker, G. A. 1992). Due to this, I discovered two distinct decline curves (exponential and gradual) with time in the theoretical fields of fixed mating regimes and extreme opposites (Fig. 2C, D). When examining how the degree of polyandry is influenced by the temporal OSR dynamics, which is a phenomenon observed in numerous other animals, these simulations would be highly informative. since a population collected on the same day showed no discernible changes in morphology or maturation status between mated and virgin females. According to Seidou, M. et al. (1990), Kubodera, T. et al. (2007), Burford, B. P. & Robison, B. H. (2020), mating signals that can convey a female's receptivity to her partner are mating signals that should be the focus of future research.

Figures

A

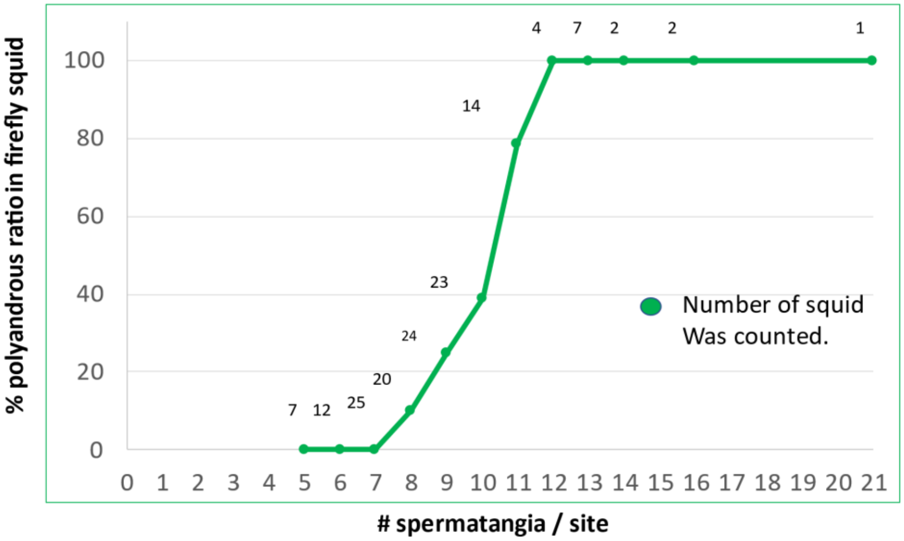

Figure 1 A The level of polyandry is correlated with spermatangium number. The percentage of polyandry and the number of spermatangia (# spermatangia/site) affixed to the seminal receptacle are correlated. I genotyped all the spermatangia attached to one of the SRs that is, the one with the greater quantity of spermatangia for every female. In each plot, the number of female individuals was labeled. As a function of the total number of spermatangia per site, the frequencies of females with varying numbers of sires are displayed as polyandrous, while those with ≤7 spermatangia were exclusively monandrous. In females storing eight to eleven spermatangia, the rate of polyandry rose as the number of spermatangia increased.

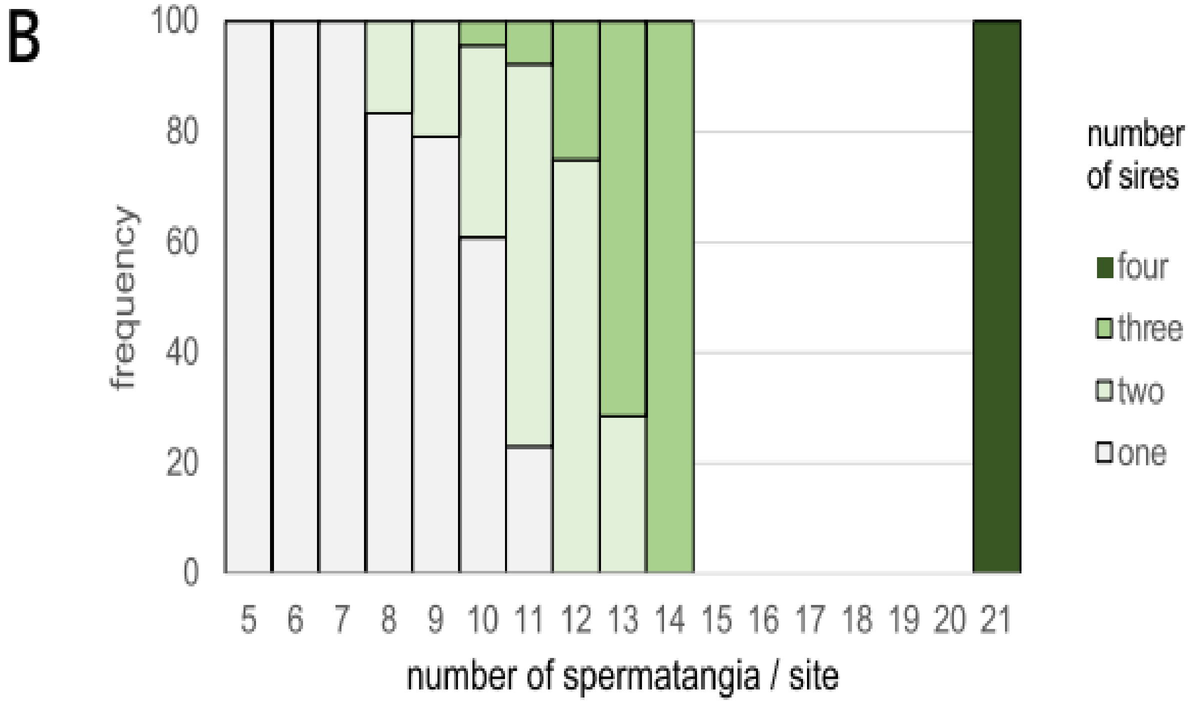

Figure 1 B The frequency distribution of females with varying numbers of sires is plotted against the total number of spermatangia at each site. The higher the spermatangium number in females with ≥8 spermatangia in the SR, the higher the sire number. These findings imply that the number of attached spermatangia can be used to estimate the likelihood of polyandrous mating.

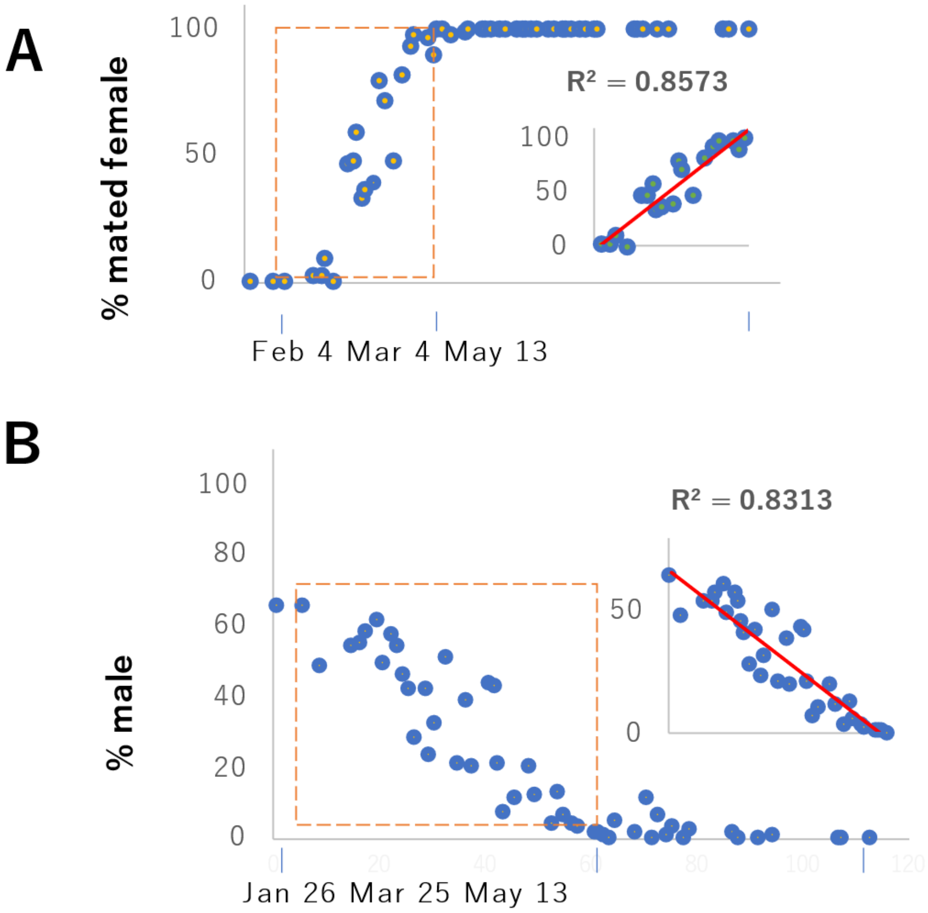

Figure 2 (A, B) Calculating the operational sex ratio in mating regimes with polyandrous and monoandrous individuals. In fig 1. A B. Seasonal variation in the proportion of non-virgin females (A) and males (B) are displayed with extracted data points for approximative linearization *(dashed red boxes, insets)*. The same plots were created using the combined data from 2019–2022.

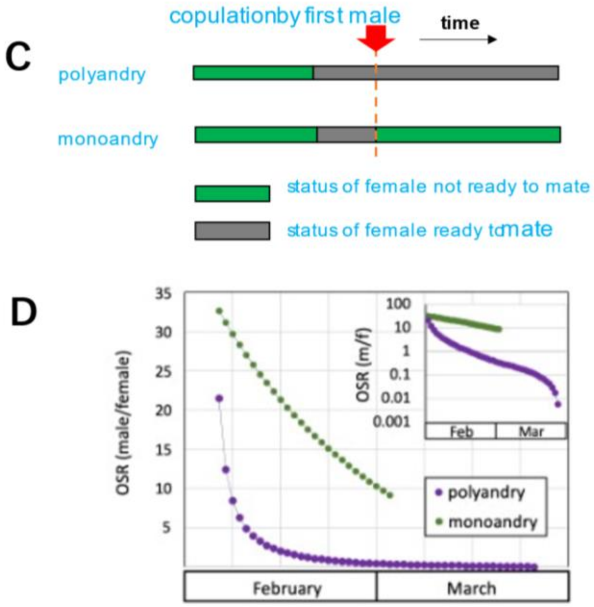

Figure 2 (C, D) Diagrammatic representation of the changes in female status under monoandrous and polyandrous mating regimes. At a specific growth point in the polyandrous mating regime, a female's status changes irreversibly from "not being ready to mate" (green box) to "being ready to mate" (grey box), at which point the female becomes more receptive to reproduction. When females in a monoandrous mating regime mate once (broken orange line with arrow), their status changes from "being ready to mate" (grey box) to "not being ready to mate" (green box). D The method is applied to calculate daily changes in OSR according to either the green plots (monoandrous) or the purple plots (polyandrous) mating regime. Its semilogarithmic scale is shown in the inset.

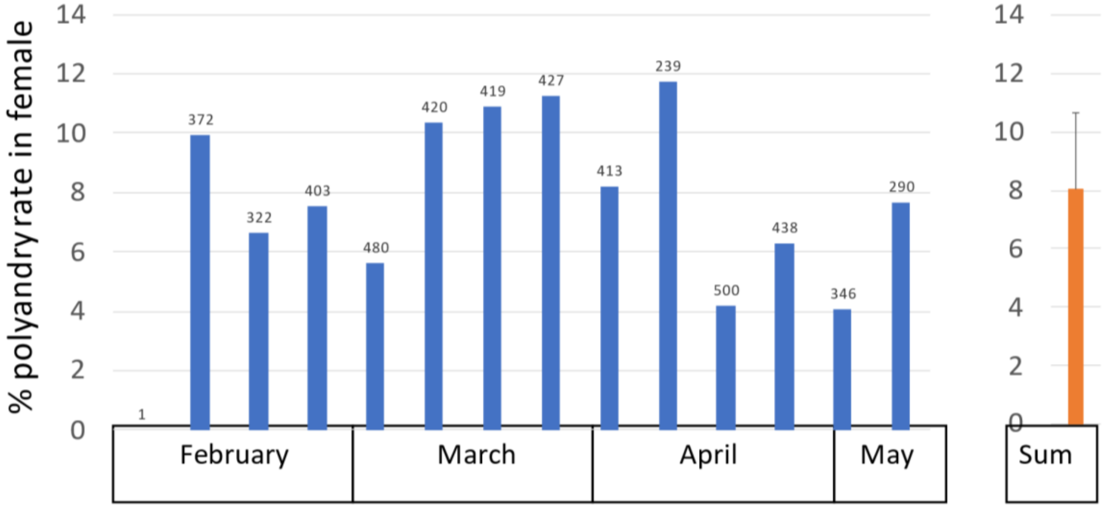

Figure 3 Seasonal dynamics of polyandry in *W. scintillans*.

I assessed the percentage of mated female populations that are polyandrous weekly (representing four columns/month) for the whole fishery season, based on the data displayed in Fig. 1A. The numbers denoted on top of every column indicate the sample size. The entirety of the polyandry percentage (mean±SEM) is presented on the right (Sum).

Acknowledgments

I thank Koichi Ozaki. I also thank Dr. Osamu Inamura, Mr. Satoshi Kusama and other members of Uozu Aquarium for sample collections and useful information about *W. scintillans*. This study was supported by Kakenhi (#21K06333 to N.H.) and the faculty of Life and Environmental Sciences at Shimane Univ. (N.H. and M.N.E.A.)

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