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Courtship and reproduction in *Carybdea sivickisi* (Cnidaria: Cubozoa)

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Abstract Courtship and fertilization events in cubozoans have received little attention from biologists, and much of what we know about these processes is based on conjecture or scant anecdotal evidence. I set out to describe these processes in the cubozoan *Carybdea sivickisi* by observing mature medusae in vitro. Mature adults engage in courtship during which spermatophores are transferred from the male to the female, who then inserts the gametes into her manubrium. Females accepted multiple spermatophores from multiple males, and only produced one embryo strand. This study also provides evidence that the presence of conspicuous velar spots on the female's bell margin is a signal of sexual maturity, and that sexual maturity was not reached in either sex until individuals had a bell diameter of at least 5 mm.

Introduction

Documenting a species' courtship rituals is an essential first step towards understanding the evolution of reproductive behaviours. Unfortunately, much more

effort has been spent describing the behaviours of so-called "complex" terrestrial and marine invertebrates (e.g. Urbani et al. 1998; Heller et al. 2000) than of the "simpler" invertebrates. One phylum that has been little studied is the Cnidaria, which contains three classes that possess species exhibiting sexual medusoid forms. Of these groups—Hydrozoa, Scyphozoa and Cubozoa—only the last group includes cnidarians showing the specialized "wedding dance" courting behaviour (described by Werner 1973).

Circumstantial evidence suggests that following courtship, the spermatophore that is transferred from the male is used for internal fertilization by the female (Werner 1973; Arneson 1976; Yamaguchi and Hartwick 1980; Hartwick 1991a). This hypothesis is largely based on Werner's (1973) report of a single courtship observation in the carybdeid *Tripedalia cystophora*, where spermatozoa were bundled to form spermatozeugmata (singular: spermatozeugma); spermatozeugmata were further bundled to form larger spherical spermatophores that were transferred from male to female (Werner 1973). An account by Hartwick (1991a) describes the presence of a red-orange spermatophore (containing thousands of aggregates of active sperm) on the tentacle of a female *Carybdea sivickisi*, who deposited an embryo strand the next day. Despite the limited nature of these descriptions, it is often cited as fact that females will insert the spermatophore into the manubrium for use in internal fertilization (Werner 1973; Harrison and Westfall 1991; Williamson et al. 1996). In the chirodropid, *Chironex fleckeri*, however, there is evidence that both internal and external fertilization occur (Yamaguchi and Hartwick 1980), suggesting that internal fertilization may not be a ubiquitous feature.

Given the anecdotal and incomplete information available on the courtship and fertilization events in cubozoans, I set out to describe these processes in the species *Carybdea sivickisi* by observing mature individuals in vitro. I also collected data on the morphology and early life-history of this cubozoan.

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Materials and methods

Study species

Carybdea sivickisi, whose taxonomic description was first given by Stiansy (1922), is a small (≤ 14 mm diameter) sexually dimorphic cubozoan, characterized by adhesive apical pads, velar spots on the females, keyhole rhopalar niches and the production of an embryo strand (Hartwick 1991a). Its behavioural patterns are often cryptic and occur nocturnally (Hoverd 1985; Hartwick 1991a; Matsumoto et al. 2002).

Sampling

Carybdea sivickisi medusae were collected between 15 May and 5 August 1996, and between 5 May and 24 June 1997, between 1930 and 0100 hours (Japan Standard Time; +9 h UTC). Water temperature was approximately 23–28°C. Medusae were collected at four sites around Okinawa, Japan (26°10'N, 127°40'E): Motubu Shinko port; in front of the Okinawa Prefectural Sea Farming Center; the Ginowan Marina and in Nakagusuku Bay. Individuals were attracted using a light, and collected with a plankton net or a hand-held scoop. A total of 126 male, 99 female and 3 juveniles were collected. Male and female *Carybdea sivickisi* individuals were reared separately in floating plastic strainers (diameter 25–30 cm) in a continuous-flow seawater system located in the culture house of the Tropical Biosphere Center Sesoko (TBSC), Okinawa. *Artemia* nauplii and various species of zooplankton collected at the time of sampling were provided as a food source to the medusae.

Additional *Carybdea sivickisi* specimens (preserved in 70% ethanol or 4% formaldehyde solution) collected between 8 May and 8 June 1994 and 1 April and 5 July 1995 were obtained from the Coral Reef Studies Laboratory of the University of the Ryukyus (Okinawa).

Morphometric analyses

Each individual was placed in a petri dish containing a drop of water, and a clear plastic millimeter grid was placed underneath. Medusae were observed through a microscope, and the diameter of the bell measured (± 1 mm). In males, the area (in mm²) of the paired gonads was calculated by measuring the area filled by pigmented structure in a single pair of gonads. Relative size of gonads in each male was calculated as a percentage of size of the bell side's surface area. Additionally, the level of gonadal development (three categories: absent, faint, conspicuous) was noted in both sexes, as were slight differences in gonad colour and tentacle banding (orange and white, red and white, mauve and white) in males (see Results).

Many *Carybdea sivickisi* females possess "velar spots", pigmented terminal regions of structures that branch off

from the palmate velar canals (four per quadrant). These spots, barely visible without the aid of a microscope, are dark-orange in colour, and are believed to be important in sexual recognition (Hartwick 1991a). In order to test this hypothesis, I recorded whether the velar spots in the females were conspicuous, faint or absent.

Courtship trials

In order to simultaneously examine courtship behaviour and mate choice in *Carybdea sivickisi* and to test for the presence of sperm competition between large and small males, I conducted 25 in vitro courtship trials using mature adults (2 males and 1 female; all with bell diameter ≥ 5 mm), which were placed into a 700-ml jar of seawater. Males were selected such that, in each trial, one individual (MA) was smaller or of equal size to the female, and one male (MB) was larger than the female. All trials were performed in a dark room between 1930 and 0400 hours, in order to simulate normal breeding conditions. Observations on courtship and mating were made with the aid of a flashlight. Individuals were used in courtship trials within 1–5 days of their collection from the field. Females that showed signs of carrying embryos (i.e. an opaque bell or cloudy gastric pockets) were not used in order to observe the production of embryo strands that resulted from trial matings. In each trial, a new group of medusae was used, except on one occasion, where males were used on a 2nd day to determine whether males that had transferred many spermatophores could replenish their sperm supplies by the next day. In total, 25 courtship trials were conducted, each lasting 2 h.

Early life-history

Following spermatophore transfer, females were held individually and monitored for embryo-strand discharge, in order to determine approximate gestation time. The mass of the resulting embryo strands, as well as that of embryo strands produced by females carrying embryos upon capture, was determined (± 1 mg). Embryo strands were reared in a 150-ml glass bowl containing filtered seawater. A watch glass or acrylic slide that had been soaked in seawater was placed at the bottom of the bowl in order to provide a substrate for planulae settling as polyps. Once polyps had reached the one- to four-tentacle stage, the polyp-covered substrate was transferred daily to a new bowl of seawater.

Results

Observations on sexual maturation in *Carybdea sivickisi*

When juvenile medusae (from the field) reached approximately 4 mm in bell diameter, individuals began

to show sexual dimorphism, and the region flanking the interradial canals showed evidence of gonadal development.

At an approximate bell diameter of 4 mm, *Carybdea sivickisi* males developed two hemispherical patches of pale-orange pigment at each interradius (eight in total). Male medusae reached sexual maturity once they had achieved a bell diameter of 5 mm, and the orange patches at the interradia developed into a pair of flattened hemispherical gonads—hemigonads—which were pinched at the perradius. These paired gonads measured on average 1.5 mm² in area, which is equivalent to 2.35% surface area of the bell side. Paired gonads contained a triangular sperm-filled structure that was red or dark-orange in colour (Fig. 1a). Following transfer of spermatophores to the female, the centre of the hemigonads appeared visibly paler. Males were phenotypically polymorphic for tentacle banding: of 126 live males captured, 92 had red and white bands, 30 had orange and white bands and 4 had mauve and white bands.

In female medusae, pairs of gonads (gastric pockets) developed at each interradius as narrow, pale sheets of tissue (Fig. 1b). Velar spots were faint in females 4 mm in bell diameter, but were clearly defined in mature females > 5 mm in diameter. Of the 99 females collected, 77 had conspicuous spots, 11 had faint spots and 11 had no visible spots. Males did not initiate courtship with females lacking velar spots, and were mostly unresponsive to females with pale spots. In the one instance courtship was observed between a male and a female with pale spots, four spermatophores were transferred but this did not produce any viable embryos. This female may have been perceived by the male as being sexually mature, as her velar spots became conspicuous the following day.

In both sexes, a pair of hemispheric sacs below each of the gastric phacellae, continuous to the gastric ostium, was observed. These gastric sacs had a dark-orange pigmentation, and contained sperm, in both males and females. This characteristic, in conjunction with their location, suggests that these sacs function as sperm-storage organs, as spermathecae in females and seminal vesicles in males.

Observations on courtship and sperm-transfer dynamics

In the *in vitro* trials, courtship began with the female and one of the males swimming around in the jar, with their tentacles fully extended. The male would then attach one of his tentacles to one of the females, gaining control of the female and pulling her around. The male then contracted his tentacle in order to bring the oral openings (manubriums) of the two individuals into direct contact. Upon contact, the area below each of the male's hemigonads darkened and a strand of red-pigmented sperm was released from the perradial puckering into the subgastric sacs. These eight strands of sperm were merged into four strands in the stomach and finally

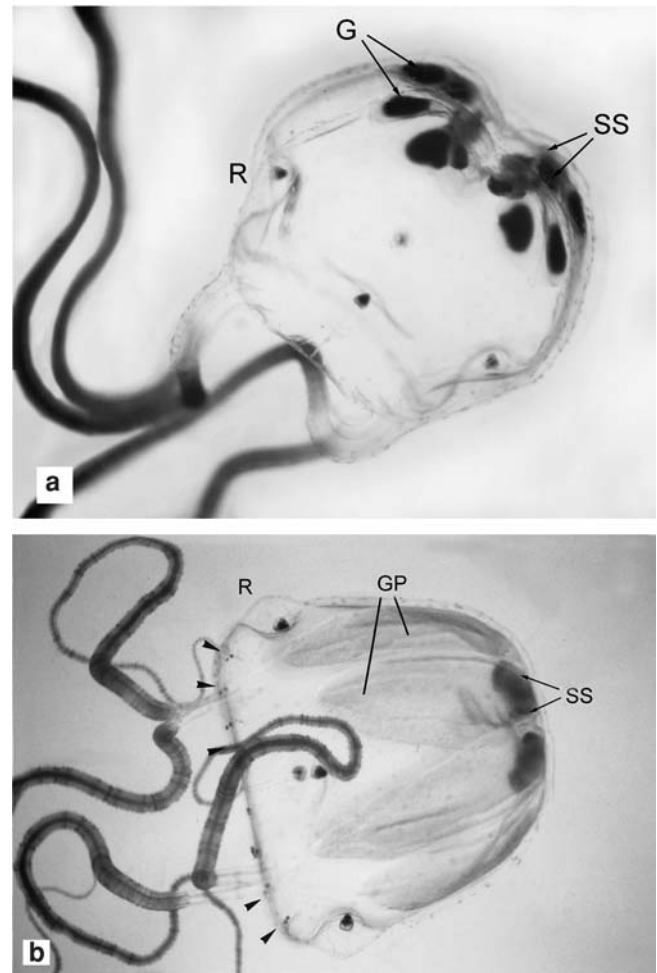


Fig. 1 Full bell lateral view of a mature adult **a** male and **b** female *Carybdea sivickisi* medusa. Labelled structures are rhopalia (*R*), subgastric sacs (*SS*), male hemigonads (*G*), gastric pockets (*GP*) which encase the female gonads, and ovaries (*O*). Arrows point to the velar spots on the female's bell margin. Both individuals have a bell diameter of 8.5 mm

coalesced into one thick strand in the manubrium (Fig. 2). In the male's manubrium, a spermatophore was formed at the bell's margin, which was then transferred to one of the female's tentacles. Following transfer of the spermatophore, the male released the female, who inserted the spermatophore into her manubrium (Fig. 3).

Observations on spermatophore-transfer frequency and use

Spermatophore transfer occurred in 18 of the 25 *in vitro* trials. In these 18 trials, a total of 75 spermatophore transfers were observed (average 4 transfers per trial, range 1–8 transfers). In the seven trials where no spermatophore transfer was observed, in most cases courtship occurred, but no spermatophores were produced despite courting, and in one or two cases, females did not extend tentacles or participate in courtship, and were generally immobile at the bottom of the jar. The greatest

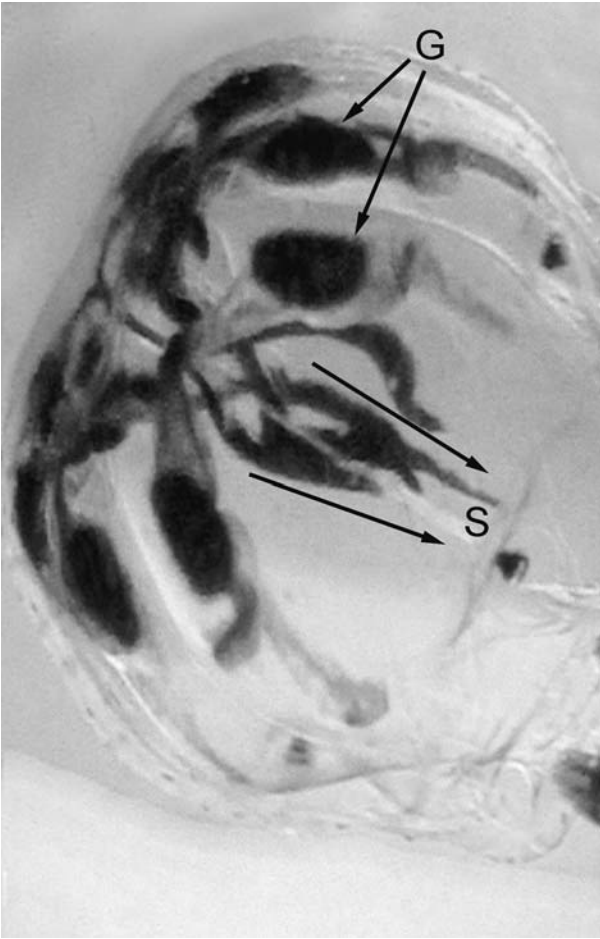


Fig. 2 Lateral view of mature adult male *Carybdea sivickisi* discharging a spermatophore during courtship. In *Carybdea sivickisi*, each of the eight hemigonads releases sperm strands that merge into four strands in the subgastric sacs. Labelled structures are male gonads (G), and spermatophore strands (S). Plate reproduced from video

number of spermatophores transferred by one male in a 2-h trial was six. With each successive ejaculation, the male's gonads became visibly paler. Despite this considerable expenditure, this male was able to quickly replenish his sperm stores, as he was capable of transferring multiple spermatophores the following day.

There was no significant difference in the number of spermatophores transferred from the smaller (MA) and the larger (MB) male to the female ($N_{MA} = 36$, $N_{MB} = 39$, binomial test $P = 0.8176$ NS). Similarly, there was no difference in the time required to complete spermatophore transfer (MA: mean 300 s, range 129–1,389; MB: mean 314 s, range 104–820 s). MA and MB males often initiated courtship with the female simultaneously. In 58 cases, this 3-way courting concluded with 1 male breaking off from the group without transferring spermatophores, while in the other 17 cases, females received spermatophore transfers from both males before she was released.

Females inserted all spermatophores that were transferred to their tentacles into the manubrium. This

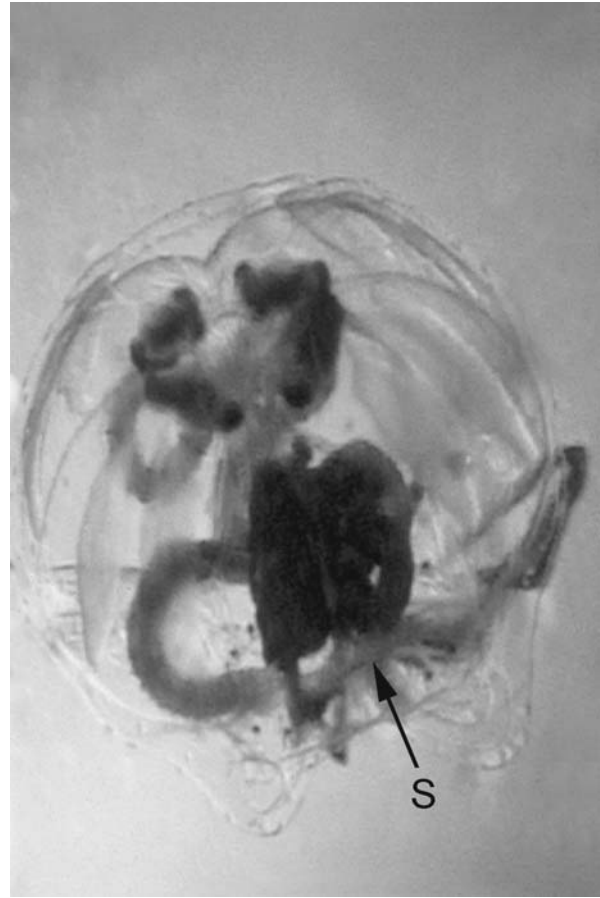


Fig. 3 Apical view of a mature adult female *Carybdea sivickisi* medusa inserting a spermatophore (S) into her manubrium. Plate reproduced from video

process took an average of 239 s (range 13–1,943 s, SD 392). When females received multiple spermatophores, the delay between transfer and insertion became progressively longer. As a consequence, males would sometimes initiate courtship with females while they were still in the process of transferring previously received spermatophores. Following insertion into the manubrium, the spermatophore was transferred into the female's subgastric sacs (putative spermathecae) after approximately 1–2 h and the gastric pockets soon became cloudy. The gestation period lasted approximately 55 h (range 44–66 h), after which time thousands of embryos began to circulate through the gastric ostia, giving the bell an opaque brown colour. At this point, the female began the process of extruding embryos by pulsating rapidly, which resulted in the embryos accumulating at the bell's four interradial. After an hour, the female exuded a gelatinous cream-coloured embryo strand (Fig. 4). Five of the 18 females that mated in the observation trials produced embryo strands, which had an average mass of 55 mg (range 10–84 mg). Each strand contained over 3,000 embryos, each approximately 100 μm in diameter. An additional 22 embryo strands were recovered from females that were apparently fertilized before their capture. The average mass of

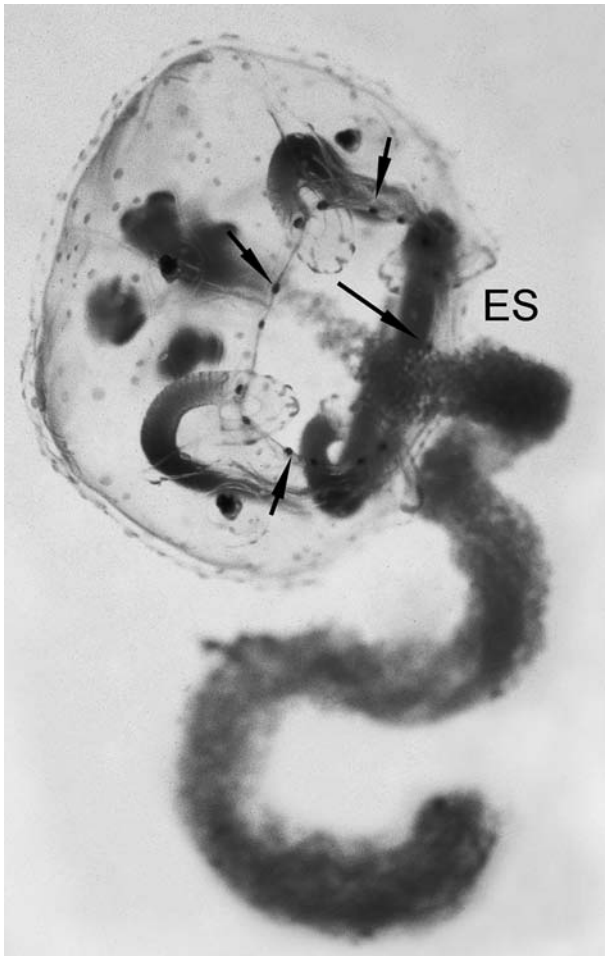
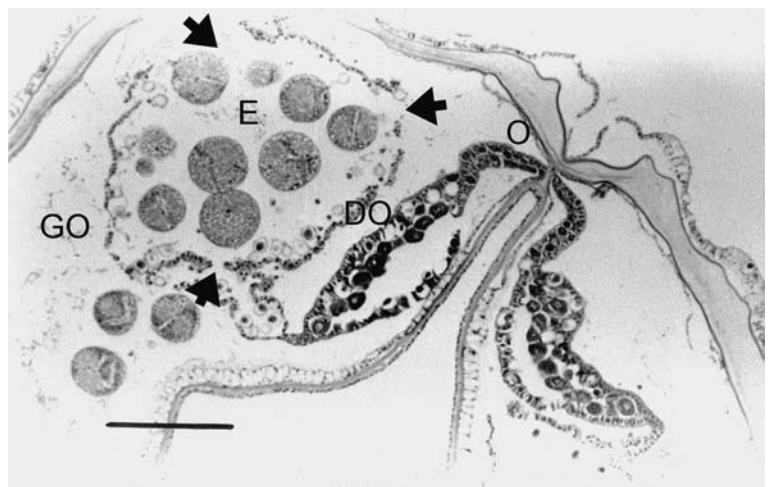
Early life-history of *Carybdea sivickisi*

Fig. 4 Oral view of a mature adult female *Carybdea sivickisi* medusa exuding a larviparous embryo strand (ES) from her manubrium. Small arrows point to conspicuous velar spots on the bell margin. Plate reproduced from video

these strands was 73 mg (range 38–100 mg), and the gestation time, estimated from the time of capture until embryo-strand production, was 39 h (range 22–57 h).

Fig. 5 Cross-section of the gastric ostium region at the interradius of a recently spent mature adult female *Carybdea sivickisi* medusa showing the ovaries (O), developing oocytes (DO) and residual embryos (E). Arrows indicate where the gastric pockets have ruptured liberating embryos throughout the gastric ostium (GO). The bar represents 100 μ m



Following deposition of the embryo strand onto the substrate by the female (day 0), the embryos began to develop. The gelatinous membrane that surrounds the embryo strand dissolved after 4.5 days, and the spherical embryos developed into pear-shaped motile planulae by day 6. The motile planulae (140 μ m in diameter) settled on the substrate by day 9.5 and developed into polyps (200–700 μ m in diameter) with 1–10 tentacles by day 14.5. The next step in their development occurred by day 17.5, when polyps elongated to become creepers, which often aggregated into long chains (> 1,000 μ m long) that appeared to function as a single unit. These observations are in accord with those made by Hartwick (1991a) on *Carybdea sivickisi*, and are similar to the known early life-histories of three other carybdeids: *T. cystophora* (Werner et al. 1971), *Carybdea marsupialis* (Studebaker 1972) and *Carybdea alata* (Arneson 1976). One notable exception is that there is no mention made in Hartwick's (1991a) report (or elsewhere in the cubozoan literature) of the aggregations seen in this study. Whether the development of these aggregations is a unique and normal characteristic of *Carybdea sivickisi* life-history, or an artefact of holding conditions, requires further study.

Histology of the female gonads and putative spermathecae

Histological analysis of a female *Carybdea sivickisi* with conspicuous velar spots (approximately 250 μ m in diameter) and a bell diameter of 7.5 mm revealed a ruptured gastric ostium, suggesting a recent discharge of embryos. Blastulae at various developmental stages (some having undergone three to four cleavages) were observed in the gastric ostium (Fig. 5). Embryos were seen within the gastric ostium and velar canals of the velarium, as well as near the opening of the sinus that connects the subgastric sacs. The ovaries exhibited a development gradient, with the most-developed oocytes

located near the gastric pockets and the least-developed oocytes located near the interradius.

Discussion and conclusions

This study contains the most comprehensive description of the courtship behaviour in the sexually dimorphic cubomedusa *Carybdea sivickisi* that has been produced to date. In vitro courtship trials were used to observe the interactions of males and females during courtship and describe spermatophore production and use. The pattern of courtship that was observed follows a sequence that is consistent with that hypothesized by Werner (1973) for *T. cystophora* and by Hartwick (1991a) for *Carybdea sivickisi*.

This study highlights the importance of velar spots as a characteristic of female sexual maturity. Only sexually mature females possessed conspicuous velar spots, and males only transferred spermatophores to females that possessed conspicuous spots. Whether the male *Carybdea sivickisi* uses these spots as a mate recognition cue, or whether another signal is used, is not clear. Cubozoans do, however, possess the most sophisticated visual system of all gelatinous zooplankton (Conant 1898; Pearse and Pearse 1978; Piatigorsky et al. 1989; Martin 2002). Further studies to determine whether the complex lens of *Carybdea sivickisi* can resolve an image on the retina, and whether coordinated vision is involved in species and mate recognition, should help resolve this question.

Spermatophore use in *Carybdea sivickisi*

After the female exuded an embryo strand and then inserted several spermatophores, no further embryo strands were exuded before she died 1–2 weeks later. This could mean that the female only produces one embryo strand during her lifetime, which is consistent with Hartwick's (1991a) report that the female *Carybdea sivickisi* medusa dies 16 days after embryo-strand discharge. Werner (1980) claimed that the lifespan of a medusa can be indirectly estimated by its seasonal presence in plankton samples. As *Carybdea sivickisi* is only present in Okinawan waters between April and July, this suggests a pattern of seasonal alternation of generations similar to that noted in the chirodropid *Chironex fleckeri* (Yamaguchi and Hartwick 1980; Hartwick 1991b). Werner (1980) also stated that most medusae have a very short lifespan that ends after sexual reproduction. More research is needed to determine whether this is the case with *Carybdea sivickisi*.

Although spermatophore transfer was observed in 18 of the courtship trials, only 5 of these *Carybdea sivickisi* females discharged embryo strands. It is very difficult to distinguish female gonads that are barely mature from those that have already extruded an embryo strand, as in both cases the gastric pockets appear as a thin outline at the interradii. Since females with conspicuous velar

spots engage in courtship regardless of their gonadal condition, there is the possibility that the low embryo-strand yield may have resulted from the inadvertent use of post-reproductive females in the trials.

Since females accepted multiple spermatophores from different males during the mating trials, it is likely that *Carybdea sivickisi* reproduction involves considerable sperm competition, and it may be a good model species for studies of paternity assurance similar to those being conducted in other marine organisms (e.g. Urbani et al. 1998). Additional studies may explore whether or not spermatophores of *Carybdea sivickisi* represent nuptial gifts used by the female for nutrition and egg production (for examples in other species, see Reinhold 1999; Heller et al. 2000).

Comparison of spermatophore production in *Carybdea sivickisi* and *Tripedalia cystophora*

The mechanism of discharge of the spermatophore in *Carybdea sivickisi* is similar to that reported in another cubomedusa, *T. cystophora* (Werner 1973). In *T. cystophora*, spermatozeugmata, brownish spermatozoan-filled spheres of various sizes and structure, are formed over 1–2 days and embedded between four groove-like structures of the gastric cavity. These spermatozeugmata migrate from the four gastric pockets through the opening in the gastric cavity, and are collected there in groove-like structures as brownish spherical spermatophores, which are transferred from the male to the female (Werner 1973). In *Carybdea sivickisi*, red-pigmented sperm bundles are produced and then stored in subgastric sacs (seminal vesicles) for a length of time yet to be determined. During courtship, the sperm are released as eight red strands, which the male forms into a single bundle as they leave the manubrium. Beyond this point, the two species differ substantially. In the case of *T. cystophora*, other than one spermatophore transfer observed by Werner (1973), following the 1–2 day “mating phase”, males and females release their “unused” eggs and sperm into the water. Conversely, all *Carybdea sivickisi* medusae spermatophores that were discharged during the 2-month trial period in 1997 were transferred and inserted by the female.

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