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Males also have their time of the month! Cyclic disposal of old spermatophores, timed by the molt cycle, in a marine shrimp

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Summary

That sexually mature females go through hormonally regulated reproductive cycles is a well-established phenomenon in sexually reproducing organisms. Males, on the other hand, are commonly regarded as being continuously ready to mate. 'Programmed sperm degradation' on a periodic basis or an innate sperm 'expiry date' have never been shown. This manuscript describes a newly discovered molt-dependent mechanism by which old sperm is periodically removed from the reproductive system of male *Litopenaeus vannamei* shrimp. Firstly, it is shown that the spermatophores of males held in captivity become progressively melanized, a process that eventually renders them impotent. Then, by using melanin specks as a color marker, it is demonstrated that this phenomenon can be delayed and even reversed as long as the males remain sexually active. Lastly and most importantly, it is shown that male shrimp go through reproductive cycles that are strictly associated with their molt cycles, which, in turn, are hormonally regulated. Intact intermolt spermatophores disappeared about 12 h premolt, and a new pair of spermatophores appeared in the ampoules the day after the males had molted. This phenomenon was observed in an almost constant portion of males, both those in an all-male population and those in mixed male/female populations, even during the times that the females of those populations were not vitellogenically active. To the best of our knowledge, this is the first report of males of any animal species exhibiting endogenous reproductive cycles, as do females, and of the finding that spermatozoa have a predetermined expiry date, a feature that may possibly contribute to male fitness.

Key words: *Litopenaeus vannamei*, shrimp, male, female, sperm, spermatophore, melanization, mating system, impotency, sterility, molt cycle, reproductive cycle.

Introduction

In sexually reproducing organisms, males generally face reproductive challenges with regard to sperm viability both before and after ejaculation. It is commonly accepted that spermatozoa are almost totally inactive before ejaculation, becoming active only thereafter (Hamamah and Gatti, 1998). Spermatophores or seminal fluids – the end-product of the male reproductive process – are held inside the reproductive tract, but topologically outside the body: they are therefore not protected by the animal's defense system and may be subject to environmental (e.g. oxidative) stress and/or the action of contaminants. Despite the potential time-related loss of sperm viability during storage in the male reproductive tract, the notion that sperm might have an inherent 'expiry date' has never been given any serious consideration. It is similarly not known whether sperm are recycled periodically in non-mating males.

In crustaceans, numerous physiological processes, including female reproduction, have been shown to be closely linked to the molt cycle (Skinner, 1985). In many decapod crustacean species, such as shrimps, lobsters and crabs, female reproduction is synchronized with the molt cycle and is thus cyclic by definition (Adiyodi, 1985; Nelson, 1991). The nature of the association between molt cycles and reproduction in decapod males is, however, not known, although a few clues do exist, all of them from penaeid shrimp: manually ejaculated *Litopenaeus vannamei* exhibited new pairs of spermatophores in their ejaculatory ducts (ampoules) only after molting (Heitzmann and Diter, 1993); a decline in spermatophore quality as the molt cycle progressed was observed in *Fenneropenaeus indicu*s (Muthuraman, 1997); and naturally mating *L. vannamei* and *L. setiferus* were shown to be carrying new pairs of spermatophores a few days after mating within the same molt cycle that the mating had occurred (Leung-Trujillo and Lawrence, 1991). Whereas these are the only reported indications of an underlying periodic reproductive mechanism

in shrimp males, another reproduction-related phenomenon, spermatophore melanization, is well documented in these animals. In captive penaeid shrimps, such as *L. vannamei*, the brown pigment, melanin, accumulates in the spermatophores, and males with heavily melanized spermatophores are sexually impotent, since they cannot ejaculate (Leung-Trujillo and Lawrence, 1987; Talbot et al., 1989; Wyban and Sweeney, 1991; Alfaro and Lozano, 1993; Alfaro et al., 1993; Perez-Velazquez et al., 2001). Melanin specks may appear in the ampoule of sub-adult males even before the appearance of a spermatophore (Parnes et al., 2004).

In an investigation of melanin deposition and spermatophore viability in relation to the presence of females in *L. vannamei* shrimps held in captivity, we observed that old spermatophores were replaced with new ones in a regular cyclic manner that was closely coupled to the molt cycle. In this manuscript, we describe, first, the progressive melanization of the spermatophores observed in our captive shrimp populations, a process that can permanently render the shrimps impotent. Then, we show, by using the melanin specks as a color marker, that melanization can be delayed and even reversed as long as the males are engaged in sexual activity. Last and most important, we show that male shrimp go through reproductive cycles that are strictly associated with their molt cycles, which are, in turn, hormonally regulated.

Materials and methods

Male *Litopenaeus vannamei* (Boone 1931) shrimp were observed in three types of population: maturing males with pre-pubertal females (field observations), adult males with adult females, and adult males alone (see Fig. 1, left). Since the cuticle of *L. vannamei* is transparent, both the presence of spermatophores in the ampoules and the degree of ovarian maturity could be assessed accurately simply by observing the animals. Melanin specks, if present on the spermatophores, were used as a color marker indicating male reproductive status.

Field observations: maturing male/pre-pubertal female population

Field observations were carried out on large maturing shrimp populations (Fig. 1A) at two aquaculture farms in Israel (Matan-Negev Shrimps, Ramat-Negev and Desert-Shrimp Inc., Kibbutz Mashabei-Sade). The shrimps were fed with commercial crustacean pellets. From each of a number of populations, 25 males and 25 females were sampled every 2 weeks and examined externally. The females in these populations were pre-pubertal and not receptive. Laboratory sub-populations for controlled observations were taken from these field populations.

Experimental system: adult male/adult female and all-male populations

Adult shrimp were sampled from the farm populations described above and transferred to an experimentally controlled seawater system situated in a greenhouse at Ben-Gurion University of the Negev, Beer-Sheva, Israel. Water quality was monitored for ammonia, nitrite, nitrate, alkalinity, oxygen, total dissolved solids and pH. Temperature was kept at 29±1°C. Photoperiod was 14 h:10 h light:dark. The shrimp were fed with an enriched diet of fresh-frozen sandworms and squid, chopped and mixed with paprika and shrimp maturation pellets. These items were fed to the shrimps in an amount of about 25% of the wet body mass (~25%=9–10% sandworms, 15–16% squid and 1–2% pellets). For individual identification, each shrimp was eye tagged with a numbered, colored plastic ring, and a white patch with the same number was attached to the cuticle with fast-drying glue. For males, the patch was attached to the carapace, and for females, to the first segment of the abdomen so that it would not obscure the developing anterior lobes of the ovary. Each day, molt exuviae were scooped out of the tank and identified, and the respective shrimps were weighed and re-tagged with a new patch on the cuticle. Male exuviae were examined for the presence of spermatophores, as was the bottom of the shrimp pool and the biofilters.

The reproduction experiment (Fig. 1B) was conducted in the first 23 weeks of the study in an experimental system that housed 36 male and 30 female adult shrimp, with average weights per shrimp of 25.4±3.5 g and 31.4±3.3 g, respectively. After ovarian maturation had been endocrinologically induced, all females were monitored daily for signs of ovarian development. Females with fully developed ovaries were captured, identified and examined for the presence of spermatophores/sperm-mass. Twice a week, all males were examined for the appearance of spermatophores. This examination was performed in direct sunlight, since the internal features of the spermatophores and terminal ampoules were not fully visible upon examination in the dark room with the aid of artificial light, especially in the last 36 h before exuviation.

At the end of the reproduction experiment, an all-male experiment (Fig. 1C) was started with nine males from the previous phase plus 45 additional males (30.4±4.0 g per shrimp). For a period of 9 weeks, the molt cycles of these males were monitored as follows. A male that had molted was re-tagged and not examined again until 8–10 days had passed after the molt event, since it was already known from the reproduction experiment that the average molt cycle is about 14 days. From then on, the animal was examined every day until it molted again. This schedule was followed so as to minimize stress but nevertheless to obtain the maximum number of observations and minimize the chance of missing an observation of the ampoules before a molt event. The results from the statistical analysis appear as mean ± s.d.

Results

General outline of the study

The major finding of this study is the fact that adult male *L. vannamei* shrimp go through molt-related reproductive cycles that include ‘programmed’ spermatophore degradation. Fig. 1

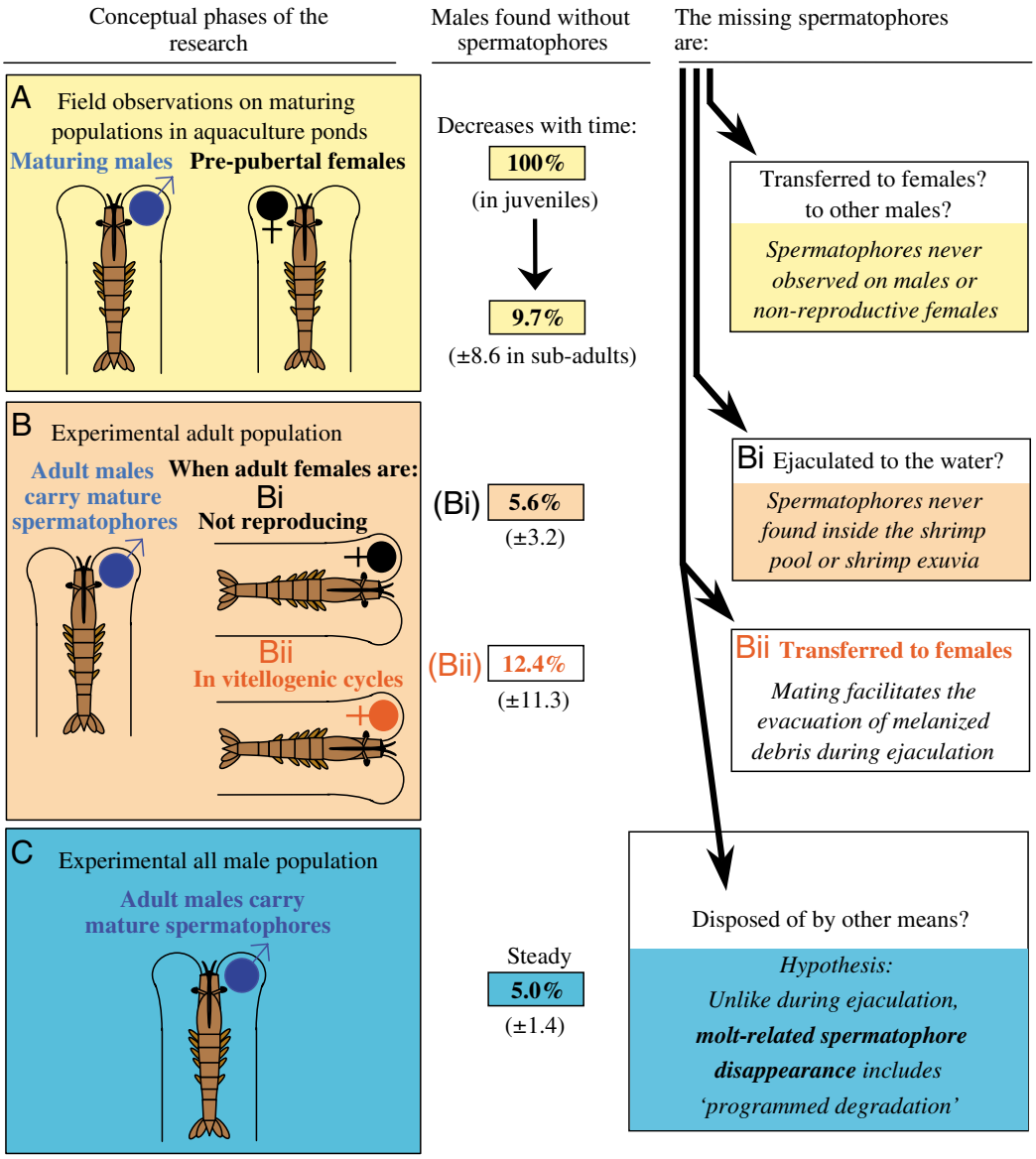

Fig. 1. Scheme showing the discovery of periodicity in the disappearance of spermatophores in *Litopenaeus vannamei*. (Left) Long-term observations on three different types of shrimp population. (Middle) The percentage of males found without spermatophores. (Right) A flow chart summarizing the logical flow of thought that led to our hypothesis. (A) Field observations twice a month on maturing populations of shrimp in aquaculture ponds showed the females to be arrested in a pre-pubertal state. The percentage of juvenile males found without spermatophores – initially 100% – decreased steadily to an average of 9.7±8.6% during a 5-month period as the animals matured to become sub-adults. The remainder of the males were found to carry mature spermatophores containing fully developed spermatozoa. (B) In an experimental adult population, males were found with mature spermatophores most of the time during which the females were not receptive, and the average percentage of males found without spermatophores during this time was 5.6±3.2% (Bi). When females were going through vitellogenic cycles and were receptive (Bii, red font), the average percentage of males found without spermatophores was 12.4±11.3. (C) In an experimental all-male population, 5.0±1.4% of the males were found without spermatophores.

outlines the entire study diagrammatically and summarizes briefly how this mechanism was discovered.

Our field observations on large maturing populations showed that the ovaries of pre-pubertal females did not mature spontaneously and that the animals did not become receptive to the males. Nevertheless, the males did mature, with the spermatophores containing mature spermatozoa. In each type of population of the current study, some of the males were found to be 'empty', i.e. without spermatophores in the ampoules. The percentage of empty males decreased with time, from 100% in juveniles to an average of 9.7±8.6% in sub-adults (Fig. 1A). In the maturing male/pre-pubertal female population, neither males nor females were found with attached spermatophores at any time.

In the experimental adult male/adult female population (Fig. 1B), melanin specks appeared on the spermatophores of

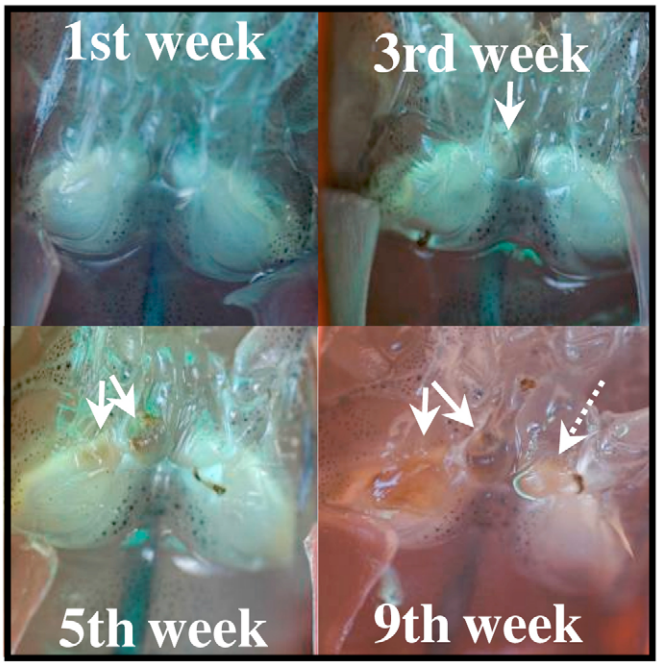

Fig. 2. Progression of melanization in a *L. vannamei* male from the mixed adult male/adult female population over a 9-week period. The pictures show the ventral side in the posterior region of the cephalothorax. First week: the spermatophores are white; third week: slight melanization (arrow) is visible in the most distal part of the left spermatophore, immediately next to the gonopore area; fifth week: melanin accumulation is clearly seen in the left spermatophore (arrows); ninth week: melanin has spread over the left spermatophore (arrows). Broken arrow indicates slight melanization in the right spermatophore.

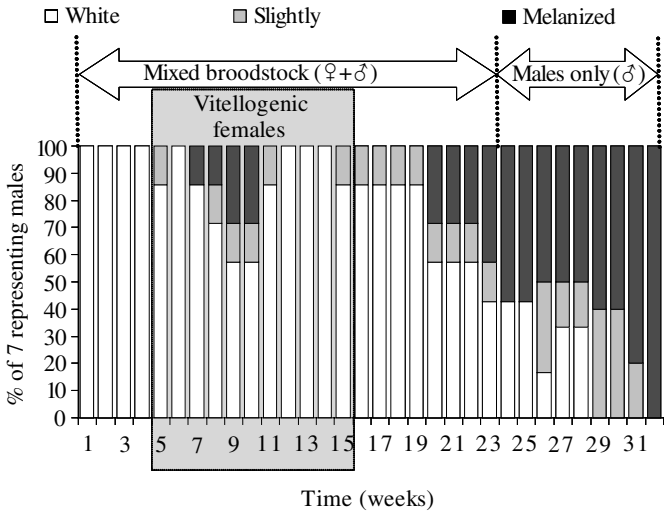

Fig. 3. Progress of spermatophore melanization in 7* representative *L. vannamei* males that were followed throughout 32 weeks, including a male-only period of 9 weeks (weeks 24–32). The light-gray rectangle that covers the histogram in the area between weeks 5–15 represents the period of time in which the females were vitellogenically active. In week 5, one of the seven males was found slightly melanized and then 'white' again on week 6. Then, three males developed melanization between weeks 7–10 but all of them recovered and were found 'white' between weeks 12–14. All the recoveries occurred strictly during the period in which the females were vitellogenically active (weeks 5–15). *Only 6 males remained at week 26 and only 5 at week 29.

some of the males about a month after the beginning of the experiment. Nevertheless, in several males the specks apparently disappeared, only to reappear after some time. This melanization phenomenon and temporary recovery from it are addressed below.

Progression of melanization and its relationship to sexual activity

Fig. 2 shows the progressive nature of melanization in a male *L. vannamei* shrimp from the adult male/adult female experimental population. At first, the spermatophores appeared to be milky-white but by the ninth week, melanin specks were present on both spermatophores. Melanization always appeared immediately next to the genital papillae as tiny light brown specks, which were clearly visible against the milky white background of the spermatophore. In most males, the specks appeared simultaneously in both spermatophores, and melanization progressed symmetrically, but in some cases it appeared initially only on one spermatophore (Fig. 2). Fig. 3 shows the progression of melanization in seven representative males from the experimental adult population that contained both males and females during the first 23 weeks of the experiment but males alone in the following nine weeks (weeks 24–32). All males sampled from the farm population had white spermatophores, but a few weeks after transfer to the experimental system several exhibited melanization in the ampoules. Of these animals, some recovered temporarily during the period in which the females were vitellogenically active (weeks 5–15) (Fig. 3). From week 16 onward, all vitellogenic activity in the females ceased, but some of the males remained non-melanized long after the female 'vitellogenic window' had closed, up to the 28th week. After that time, the spermatophores of all seven shrimps became melanized.

A better understanding of the relationship between reproductive activity and disappearance of melanization can be reached from examining the effects of artificial *in vitro* ejaculation (Fig. 4), when it can be seen that the accumulated melanin is evacuated from the ampoule together with the spermatophores.

Spermatophore dynamics along the molt cycle

Before and after the above-mentioned 'vitellogenic window' (weeks 1–4 and 16–23; Fig. 3), i.e. when the females were not reproductively active, 5.6±3.2% of the males were found to be 'empty' (Fig. 1B1) – all on the last day of their molt cycle. By contrast, within the 'vitellogenic window', a higher percentage of males, 12.4±11.3%, were found 'empty', some of them being in the middle of their molt cycles (Fig. 1B2). This

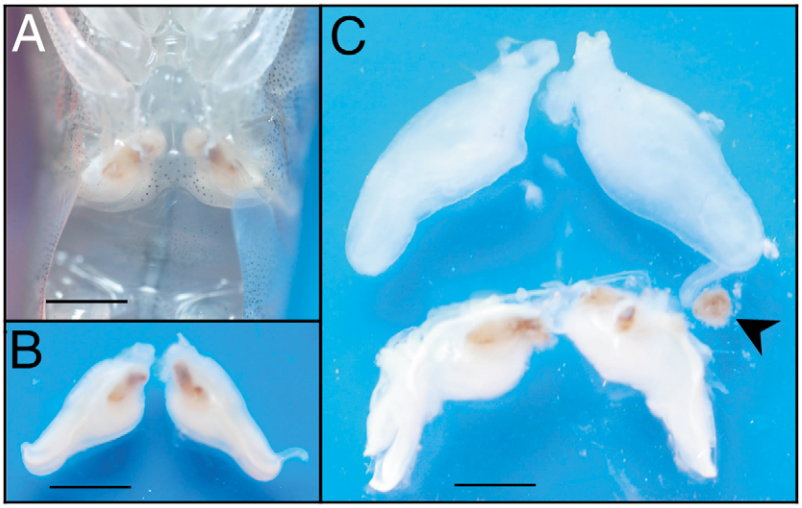

Fig. 4. Partially melanized *L. vannamei* male, and dissected components of the distal part of its reproductive system. (A) Ventral view of the intact male. Symmetrical dark brown melanin spots are clearly visible through the cuticle near the genital papillae. (B) The dissected ampoules with intact spermatophores. Here, the muscular ampoules with their intact spermatophores are stretched and turgid. (C) The same ampoules (top) after the spermatophores (bottom) had been dislodged from them by applying a gentle pressure on the edge of the ampoules opposite to the gonopore, mimicking ejaculation. The empty ampoules then lost their turgidity and became flaccid and slack. Note the melanized flake that separated out from the right spermatophore (arrowhead). Scale bars, 5 mm (A,B), 3 mm (C).

observed difference, which seems to be related to the vitellogenic activity of the females and the molt stage of the males, is discussed in greater detail below.

Fig. 5, which refers to the same adult male/adult female experimental population shown in Fig. 3, shows spermatophore dynamics in relation to the period during which the females matured and were receptive to males (pink rectangle, marking weeks 5–15, same as the gray rectangle in Fig. 3) and in relation to the percentage of molting males per night (filled circles in Fig. 5). It is clear that males (N=12) found to be 'empty' in the middle of the molt cycle (red bars in Fig. 5 and Fig. 1B2) were observed only during the time that the females were sexually receptive and all of them corresponded with cases of females that were found with spawned ovaries the day after they had been observed with fully vitellogenic ovaries. For three of the 12 males found to be 'empty' in the middle of the molt cycle, the following sequence of events was recorded: The male molted, and 24 h after the molt white spermatophores were visible in the ampoules. Then, two to three days thereafter no spermatophores were visible, since the shrimp had probably mated, but three to five days later, still within the same molt cycle, white spermatophores were again observed. The remaining nine cases were not sampled again until their next molt, after which all of them exhibited white spermatophores. Thus, these 12 cases were most probably ejaculations that took place during mating and are referred to as ejaculation-related spermatophore disappearance.

Cases of males found to be 'empty' about 12 h before they molted (number of observations = 105, green bars in Fig. 5) were observed throughout the 32-week period of the experiment, irrespective of the presence or absence of females; these cases are referred to as molt-related spermatophore disappearance (note the double-headed arrows in Fig. 5 and Fig. 1Bi,C). It was possible to record some of these cases in consecutive molt cycles, i.e. in two (N=11), three (N=2), four (N=4) and even five (N=4) molt cycles. Since the molt cycle in adult *L. vannamei* shrimp follows a very regular pattern, it was to be expected that the average percentage of males molting each night would also follow a steady pattern (black circles in Fig. 5). The percentage of 'empty' males also followed the same general pattern (green bars in Fig. 5), except for the peaks when the females were receptive (sum of red and green bars, inside the pink rectangle, Fig. 5). This percentage was similar in the mixed and all-male populations (5.6% and 5%, respectively, in Fig. 1Bi,C), but was slightly smaller than the

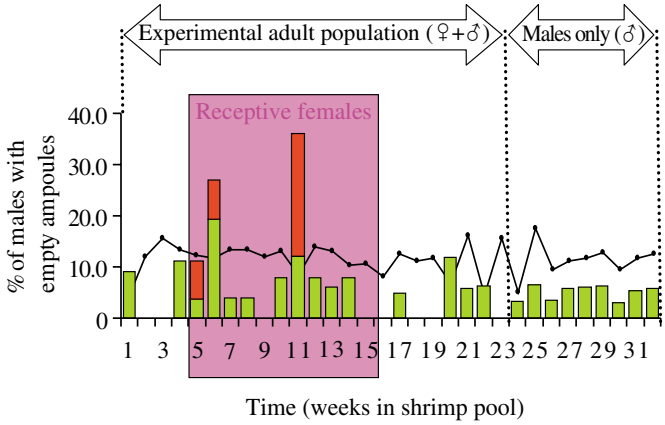

Fig. 5. Spermatophore dynamics in the experimental adult male/adult female and all-male shrimp populations in relation to the period during which the females matured and were receptive to males (pink rectangle, weeks 5–15). Time is shown on the x axis in weeks. The y axis gives the percentage of males that were found with empty ampoules at a certain week. Black circles indicate the average daily percentage of molting males out of the total number of males in the population. Green bars represent males with empty ampoules on the night of ecdysis, before they molted ('molt-related'). Red bars represent males with empty ampoules in the middle of the molt cycle ('sex-related'). It is clear that the latter were found only during the period that the females were sexually receptive. On the other hand, males with empty ampoules that were about to molt were found throughout the observation period, irrespective of the presence of females, since such males were also observed in the all-male population.

average percentage of molting males per night (7.3±5.7% and 7.0±4.8% in the mixed and all-male populations, respectively). This discrepancy is due to the fact that we were able to record all molting events but not all spermatophore disappearance events.

Three lines of evidence suggest that the spermatophores that disappeared in relation to molt events were degraded and not ejaculated. First, no spermatophores were found on the tank bottom, in the water filters or in the old exuvia that were scooped out from the maturation tank every day. Second, in 43 males examined before the molt, the ampoules contained small white lumps that seemed to be the remains of incompletely degraded spermatophores. Such partially degraded spermatophores were not observed in males that had ejaculated during mating. Third, the persistent melanization patterns in non-sexually active males differed from those of ejaculating males, whose ampoules were cleared of their contents, including melanin debris, as shown in Fig. 4C, bottom.

In 45 males with slightly melanized spermatophores, the melanin specks were clearly visible when the spermatophores filled the stretched ampoule. In these cases, unlike the sexually related recovery from melanization, melanized fragments of the spermatophore probably remained inside the ampoules, even as the spermatophores were degraded. When the emptying ampoules shrank and retracted from the cuticle, those fragments remained visible, but only faintly. Then, after the male had molted and new spermatophores had appeared, the ampoule became stretched and the melanized fragments were again clearly visible through the cuticle.

The last line of evidence comes from five males examined in late premolt apolysis (according to their pleopods and uropods) that did not have spermatophores visible through the cuticle at the time they were due to molt (according to the observation logbook). On dissection, the ampoules of these animals were found to be empty and flaccid, as expected and described above (Fig. 4).

Thus, in this study it was found that spermatophores periodically disappeared from the terminal ampoules of males during the 24 h premolt and that new spermatophores appeared after the exuviations. Fig. 6A shows the white spermatophores of a representative intermolt male shrimp observed through the cuticle. Fig. 6B shows the same shrimp about 12 h premolt. The muscular ampoules, which are visible as two lumps underneath the cuticle, do not contain spermatophores. The animal was apparently about to molt, according to the loss of rigidity of the cuticle and the state of the pleopods. Following the molt, a new pair of white spermatophores that had reached the ampoules on the night of ecdysis could be seen through the cuticle (Fig. 6C). The lower part of Fig. 6 shows three representative male time tracks, each describing four consecutive molt cycles in which the spermatophore disappearance phenomenon was observed.

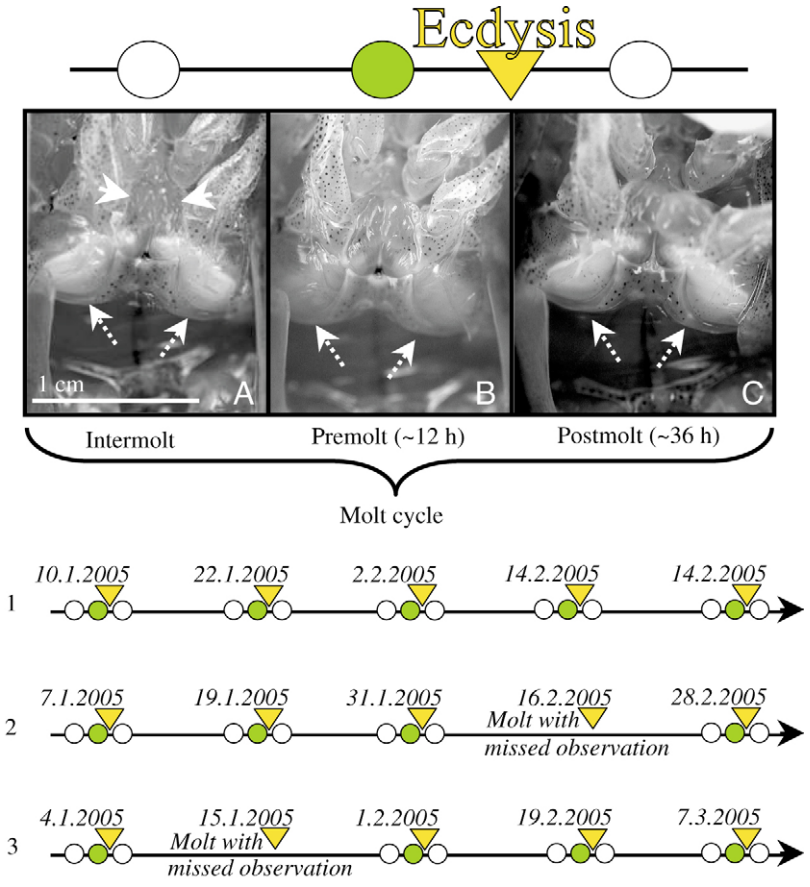

Fig. 6. Periodic disappearance of spermatophores in *L. vannamei* males related to the molt cycle. (A–C) The spermatophore disappearance phenomenon in a representative male shrimp. Above the photographs is a colour-coded diagram; the white circles indicate the presence of spermatophores in the ampoules; the green circle, empty ampoules without spermatophores; and the yellow triangle, the molt event (ecdysis). In all three pictures, the ampoules are marked with broken arrows. (A) Intermolt with intact spermatophores. Solid arrows indicate the genital papillae that house the sexual openings. (B) ~12 h before ecdysis in late-premolt; the ampoules are empty but are visible as two lumps underneath the cuticle. (C) ~36 h post molt; a new pair of spermatophores can be seen through the cuticle. The lower part of the figure shows three representative individual male time tracks, which are color coded as described above. Each of these time tracks describes five observed molt events that cover four complete molt cycles in which the spermatophore disappearance phenomenon was observed. Two missed observations, for which we did not manage to observe the ampoules before the animal molted, are also indicated. Nevertheless, in both cases the animal molted on the same night.

Discussion

In this study, 12 cases were recorded of males that were found without spermatophores in the middle of their molt cycles, which corresponded to cases of females that were found after spawning. A similar phenomenon was observed by other researchers in matings of *L. vannamei* and *L. setiferus* (Leung-Trujillo and Lawrence, 1991). The disappearance of melanin in some of the shrimp in this study is most probably the result of spermatophore evacuation from the ampoules during mating. This can efficiently remove melanin debris from the ampoules, and indeed in this study recovery of males from melanization was observed exclusively during the period that there were vitellogenic, receptive females in the population. The fact that spermatophores of sexually active males remain white is probably considered common knowledge and has been referred to only by two studies other than the current study (Leung-Trujillo and Lawrence, 1991; Browdy et al., 1996). Manually or electrically ejaculating the spermatophores can also remove melanin debris from the ampoules, and many studies use these methods to keep males in good condition in the absence of females. Indeed, most of these studies reported that, over extended time periods, spermatophores did not show deterioration (Leung-Trujillo and Lawrence, 1991; Alfaro, 1993; Alfaro and Lozano, 1993; Heitzmann and Diter, 1993; Pratoomchat et al., 1993; Alfaro, 1996; Pascual et al., 1998; Diaz et al., 2001; Ceballos-Vazquez et al., 2004). If the spermatophores are not evacuated (manually, electrically or through mating), they may become melanized in some individuals even within few days (as in Fig. 2) and subsequently hardened enough to prevent their ejaculation (Leung-Trujillo and Lawrence, 1987; Talbot et al., 1989; Alfaro et al., 1993; Perez-Velazquez et al., 2001). All these studies thus suggest that penaeid male readiness for the next copulation might actually be increased through the previous copulation.

We now need to ask whether male shrimp can maintain their readiness for copulation when there is a shortage of receptive females. Generally, the percentage of 'empty' males found in this study at any given time clearly depended on the maturational status of the males, decreasing from 100% in juveniles to about 5% in adults (see Fig. 1). Ceballos-Vasquez et al. (Ceballos-Vasquez et al., 2003) similarly reported that the percentage of 'empty' males decreased from juvenility to adulthood, but they did not explain the fact that 2.1% of their adult males were found to be 'empty' although the population under observation was a non-breeding population. In the current study, the only time that this seemingly basal level of 'empty' adult males was found to be higher was during the 'vitellogenic window' of the females, i.e. when adult males were able to mate with receptive females. At no time, were males found carrying attached spermatophores, ruling out homosexual activity, and females were never found with attached spermatophores outside the 'vitellogenic window'. In addition, there was no evidence that intact spermatophores were ejaculated through means other than sexual activity. The possibility that ejaculated spermatophores could have been eaten by the shrimp, and thus not found on the tank bottom or water filters, was not ruled out completely in this study. However, this explanation also required us to assume that the shrimp engage in a kind of regular ejaculation and such a phenomenon was never reported from shrimp studies. All the evidence suggested that when a male was found 'empty' in the absence of receptive females in the population, exuviation was imminent and would occur in less than 12 h. Thus, it was hypothesized that in such cases the spermatophores underwent 'programmed degradation', which was strictly molt related (see Fig. 1, right). Unlike ejaculation-related spermatophore disappearance, which depends on the presence of receptive females, molt-related spermatophore disappearance is an endogenous, cyclic phenomenon that occurs in all mature *L. vannamei* males at all times. This phenomenon involves the disappearance of the spermatophores during the 12 h before molt and their reappearance immediately after the molt. It probably accounts for the fact that males were observed with white spermatophores (some of them for quite a long time), even in the absence of females. We therefore propose that spermatophores have an 'expiry date' and that their average life time in *L. vannamei* matches the duration of the molt cycle.

Some clues to the timed regulation of spermatophore behavior during storage within the male have indeed appeared in the literature. In *Melicertus* (formerly *Penaeus*) *kerathurus*, a complete spermatophore is accommodated in the terminal ampoule while another is simultaneously forming in the medial vas deferens (Malek and Bawab, 1974a; Malek and Bawab, 1974b). Judging from the small variability in the dimensions of the medial vas deferens, Ro et al. (Ro et al., 1990) concluded that the migration of a new spermatophore along the sperm duct does not occur at random but is under precise regulation. In other animals, almost all the current knowledge on sperm viability, longevity and storage duration, relates to the time after ejaculation while the sperm cells are inside the female reproductive tract (Birkhead and Moller, 1993; Parker, 1970; Saunders, 2002). However, in some seasonal animal species, in which the males are known to store sperm for long periods of time, it has never been shown that the sperm is recycled during storage, and in these cases the fertilizing sperm can be several months old (Mann, 1984).

Flushing out seminal fluid or evacuating spermatophores from the reproductive tract on a regular basis could serve as a mechanism that ensures sperm viability. Whether masturbation in primates (Baker and Bellis, 1993; Starin, 2004) or sperm leakage (Cooper, 1999) represents such examples is still open to question. An isolated male cricket was seen to push out the spermatophore after grooming his abdominal tip onto the substrate, and it was suggested that in the absence of a partner, such an action might be profitable in that spermatozoa could be perpetually renewed before they became obsolescent (Sakai et al., 1991). In the many species of the Myriapoda, Arachnida and some of the more primitive groups in the Insecta (e.g. Collembola), spermatophores are extruded onto a substrate, from where they are collected by the female (Mann, 1984). However, these cases represent specific reproductive strategies,

and in all of them sperm is evacuated from the body in contrast to the mechanism reported here in which the sperm is replaced while inside the body of the male shrimp.

The regular replacement of 'old' sperm probably involves acellular-matrix degradation processes and phagocytosis of the spermatozoa. In echinoderms, phagocytosis of spermatozoa remaining in the testicular lumen after the completion of the spawning season has indeed been observed (Chia and Bickell, 1983). In the Myriapoda, it is possible that the cells of the vasa deferentia may be either secretory or phagocytic in function, depending upon the time of the year and/or the animal's reproductive cycle (Reger and Fitzgerald, 1983). In celibate human males, aging spermatozoa undergo intraluminal degeneration in the distal epididymal lumen, but the rare reports of spermiophagy by epithelial cells along the post-testicular tract do not provide evidence for wholesale removal of sperm by this mechanism (Cooper, 1999). Cyclic reproductive readiness is usually associated with female life histories (Campbell et al., 1999): females have been shown to go through reproductive cycles in many animal species ranging from chelicerates (Taylor and Chinzei, 2002) through crustaceans (Adiyodi, 1985; Nelson, 1991; Wilder et al., 2002) and insects (Attardo et al., 2005) to fish, amphibians (Polzonetti-Magni, 1999), reptiles, birds (Williams, 1999) and mammals. There are, however, also cyclic reproductive processes in males, most of them in species with a circannual cycle that includes a particular reproductive period during the course of the year. Although these cycles are hormonally regulated, they are also strictly environmentally mediated through temperature and day length changes, as has been observed in some cnidarians (Fautin, 1999), most echinoderms (Byrne, 1999), fish (Koob, 1999), most reptiles (Gist, 1999), birds (Williams, 1999), and some long-lived mammals (Zucker and Prendergust, 1999). Male insects do not produce gametes cyclically, but appear to be ready to mate at any time (Nijhout, 1994). However, in some lepidopteran insects release of sperm from the testes and secretion of a carbohydrate-rich material from cells of the upper vas deferens exhibits a circadian rhythm (Gillott, 1999). Despite of the above data regarding cyclic reproductive phenomena in males, Saunders (Saunders, 2002) concluded that: "*Rhythmicity in spermatogenesis appears not to have been examined. The absence of studies may well be related to the enduring fascination of biologists with the female system, which is usually regarded as more important to reproductive success as well as being morphologically more impressive. Indeed, much less is known of the physiology of the male reproductive system than of the female.*" This statement is also true for crustaceans, but if strictly hormonally regulated reproductive processes are to be characterized, it is from this group of animals. A unique feature of crustaceans, at least in species with indeterminate growth, is that to grow they have to molt on a regular basis throughout their lifespan. Reproduction and molting are hormonally coupled in a variety of ways (Adiyodi, 1985; Meusy and Payen, 1988; Nelson, 1991). A close link between the molt and vitellogenic cycles has been demonstrated in the female of the caridean species *Macrobrachium rosenbergii* (Meusy and Payen, 1988; Wickins and Beard, 1974). In some brachyuran (Cheung, 1969; Kurup and Adiyodi, 1981) and astacidean (Nelson, 1991) species, reproductive cycles are completed within a single intermolt period with marked seasonality. In some astacidean (Aiken and Waddy, 1980; Barki et al., 1997), brachyuran (Cheung, 1969) and caridean (Wickins and Beard, 1974) species, reproductive cycle or egg incubation may delay ecdysis and lengthen the molt cycle. In penaeids, resources are more or less simultaneously utilized for weight gain and reproduction throughout the molt cycle (Adiyodi, 1985). Penaeid females may spawn several times during a single molt cycle, but if they do not spawn, the oocytes are resorbed before molting takes place (Emmerson, 1980; Emmerson, 1983; Quinitio et al., 1993; Raviv et al., 2006).

Males, as opposed to females, are currently viewed as continuous breeders. To the best of our knowledge, this is the first study to describe males that go through periodic sperm replacement episodes when not sexually active. This degradation process could be induced by the premolt peak in ecdysteroids, but it seems that it is not as efficient as ejaculation in removing melanized debris from the ampoules.

Ejaculation-related spermatophore disappearance apparently offers active males a chance to recover from melanization while in captivity, whereas the process responsible for 'programmed degradation' does not remove melanin debris, resulting in the progressive melanization patterns observed in this study. The adaptive value of this mechanism seems to be clear, since there are no reports of melanization of the sperm duct in animals in the wild (King, 1948; Lindner and Anderson, 1956; DeLancey et al., 2005), although melanization of the cuticle and gills in wild shrimp has been observed (?. DeLancey, personal communication). Moreover, there is only one report of a single wild-caught *Litopenaeus* sp. specimen with melanized spermatophores (Chamberlain et al., 1983). In addition, this mechanism grants the male a periodic opportunity to replace old spermatophores with new ones if mating has not taken place. In *L. vannamei*, molt-coupled spermatophore disappearance is not merely the result of the reproductive system being 'switched off', like that of seasonal organisms, but the active degradation of old spermatophores before new ones occupy the ampoules. Based on our recent study of molt-related cyclic vitellogenic activity and *Vg* gene expression in *L. vannamei* females (Raviv et al., 2006) and the current study on molt-related cyclic spermatophore degradation in *L. vannamei* males, it is clear that in these organisms no phenomenon can be separated from the molt cycle and examined in isolation. Because the reproductive organs of males and females develop from essentially the same embryonic tissue, one cannot escape the similarity in the cyclic nature of these systems in *L. vannamei*. Generally, a male animal with a relatively long life span would benefit from a maintenance mechanism that keeps its reproductive tract in good condition, but whether the mechanism suggested by the present study is unique to litopenaeid shrimp or can be found in other animals is an open question.

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