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Animal behaviour

Spermatophore consumption in a cephalopod

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An individual's gametes can represent a nourishing food source for a manipulative mate. Here, we provide evidence of ejaculate and sperm consumption in a cephalopod. Through labelling male spermatophores with ^{14}C radiolabel, we found that female squid, *Sepiadarium austrinum*, consumed the spermatophores of their partners and directed the nutrients received into both somatic maintenance and egg production. We further show that in this species—where fertilization occurs externally in the female's buccal cavity—sperm storage is short-term (less than 21 days). The combination of female spermatophore consumption and short-term external sperm storage has the potential to exert strong selection on male ejaculates and reproductive strategies.

1. Introduction

Despite ejaculate consumption being well documented in nature [1–4], actual sperm consumption is rare. Where it has been found, males transfer their ejaculates to females via either placement of packaged sperm (spermatophores) on the cuticle [5,6], or direct deposition into the reproductive tract [7–10]. Once transfer is complete, females may consume a portion of these ejaculates post-copulation. Importantly, in all of these cases, sperm enter the female reproductive tract for later egg fertilization, thus ensuring that males are able to secure some fitness gains. More generally, internal fertilization appears ubiquitous across all species where any form of ejaculate consumption has so far been reported (see reviews by Vahed [11] and Gwynne [12]). By contrast, little is known about ejaculate consumption in externally fertilizing species where eggs are fertilized out of the female's reproductive tract.

The southern bottletail squid, *Sepiadarium austrinum*, is a small, solitary species native to the southern coast of Australia. Unlike many cephalopods (reviewed in [13]), females of this squid species do not possess a protected or internal seminal receptacle for sperm storage. Instead, all sperm remain inside everted spermatophores (spermatangia), which are stored externally on the female's buccal cavity, a fleshy membrane ventral to the mouth (also known as the buccal membrane, [14]). The lack of a seminal receptacle in the buccal membrane means females are unable to store sperm released from spermatangia. Preliminary observations suggest females are capable of feeding on the spermatophores passed to them by males during copulation, with such consumption occurring for up to an hour post-copulation (see the electronic supplementary material, movie S1). Furthermore, external fertilization could reduce the longevity of stored sperm compared with internally fertilizing cephalopod species, many of which store sperm for many months [15,16]. Such a combination of spermatophore consumption and short-term sperm storage is likely to exert strong selection on male ejaculate and mating strategy evolution. Here, therefore, we aimed to investigate (i) how females use the

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Figure 1. Mating pair of southern bottletail squid (photo credit: Kade Mills)

nutrients of spermatophore consumption and (ii) the period of sperm storage in females.

2. Material and methods

(a) Study species

The southern bottletail squid is an annual species with a highly synchronized breeding season and no generation overlap. Juveniles first appear in January and reach maturity by early May (indicated by the presence of spermatophores in males and spermatophore storage in females); however females do not develop mature eggs until mid-June. Individuals mate repeatedly from May until November, with all adults dying by December. During copulation (see figure 1 and electronic supplementary material, movie S1), males place numerous spermatophores in the female's external buccal cavity. These spermatophores evert to form bulbous spermatangia, which attach to the buccal cavity via a cement body. External fertilization occurs by females extracting mature eggs from their mantle (body) and passing them against the stored spermatangia in the buccal cavity before placing them on the substrate.

(b) Squid collection and housing

Juvenile bottletail squid were collected by scuba from Port Phillip Bay, Australia in early April 2011. All squid collected were known to be virgins, with individuals not reaching sexual maturity until May. Squid were housed individually in 5 l tanks and fed a diet of amphipods and *Palaemonetes* shrimp ad libitum until sexually mature. Tanks were continuously replenished with fresh sea water via a flow-through marine water system.

(c) Experimental procedures

(i) Nutrient uptake from spermatophore consumption

To assess how females incorporated the nutrients from spermatophore consumption into their tissues, females were mated either with males labelled with ^{14}C-labelled amino acids (Perkin Elmer, [^{14}C(U)]-L-amino acid mixture, product code: NEC445E050UC) or unlabelled males.

To achieve this, we first depleted the spermatophore stocks of males ($n = 6$) by mating them to exhaustion, that is, until they refused to mate with three successive virgin females. This stimulated the production of new spermatophores. These males were then fed either radiolabelled (^{14}C) or non-labelled amphipods until satiated, then isolated for two weeks to allow for the generation of new spermatophores ($n = 3$ radiolabelled and three control males). After two weeks, two sexually mature virgin females were mated with each male resulting in six labelled and six unlabelled females. Visual observation confirmed successful spermatophore transfer and spermatophore consumption by females during all copulations (see the electronic supplementary material, movie S1).

After mating, females were housed for two weeks to allow for the nutrients received from spermatophore consumption to be incorporated into their tissues. All individuals were then euthanized in a 5 per cent $MgCl_2$ sea water solution and frozen at $-20°C$. Samples of somatic and reproductive tissues were subsequently dissolved (in 1 ml 200 mM NaOH and 9 ml scintillation fluid) and the amount of radioactive label measured using a scintillation counter.

(ii) Period of female sperm storage

To determine how long female squid store sperm, we mated sexually mature virgin females with two males, with a 30 min rest period between each mating ($n = 17$ females and 34 males). Visual observation of successful spermatophore transfer confirmed that all females received and consumed spermatophores from both copulations. Females were then housed individually and permitted to lay eggs. Any clutches laid were counted for egg number and hatched in order to determine fertilization success. After 21 days, five females that had yet to lay eggs were dissected and their buccal cavities examined for the presence of spermatangia.

3. Results

(a) Nutrient uptake from spermatophore consumption

^{14}C radioactivity levels of females ($n = 6$) mated with non-labelled males did not differ from background level control vials ($n = 10$; independent t-test: immature eggs $t = 0.119$, $p = 0.907$; mature eggs $t = 0.146$, $p = 0.886$; nidamental (reproductive) glands $t = 0.267$, $p = 0.793$; digestive gland $t = 0.099$, $p = 0.922$; mantle $t = 0.458$, $p = 0.654$; electronic supplementary material, table S1). However, all internal tissue samples taken from females that mated with labelled males contained higher ^{14}C-radiolabel than unlabelled females (Mann–Whitney U-test: immature eggs $U = 31$, $p = 0.037$; mature eggs $U = 36$, $p = 0.004$; nidamental (reproductive) glands $U = 34$, $p = 0.01$; digestive gland $U = 36$, $p = 0.004$; mantle $U = 28$, $p = 0.109$; figure 2*a*; electronic supplementary material, table S1). All values remained significant after false discovery rate correction for multiple comparisons [17]. This suggests that females incorporate the nutrients from spermatophore consumption for use in somatic growth [18]. As fertilization is external in this species, the presence of ^{14}C radiolabel in the ovarian eggs indicates that the nutrients derived from spermatophore consumption most probably contribute to unfertilized egg development [18].

(b) Period of female sperm storage

Female sperm storage in southern bottletail squid appears to be limited to less than 21 days in the laboratory (figure 2*b*). First, none of the five females euthanized for buccal cavity examination on day 21 had successfully retained any of the spermatangia passed to them during copulation. Second, fertilized egg clutches were only produced within 21 days of copulation ($n = 4$ clutches from two females). Although five other females laid eggs after this time, none were fertilized ($n = 12$ clutches, figure 2*b*). The remaining females ($n = 5$) failed to lay eggs. There was no difference in clutch size when comparing

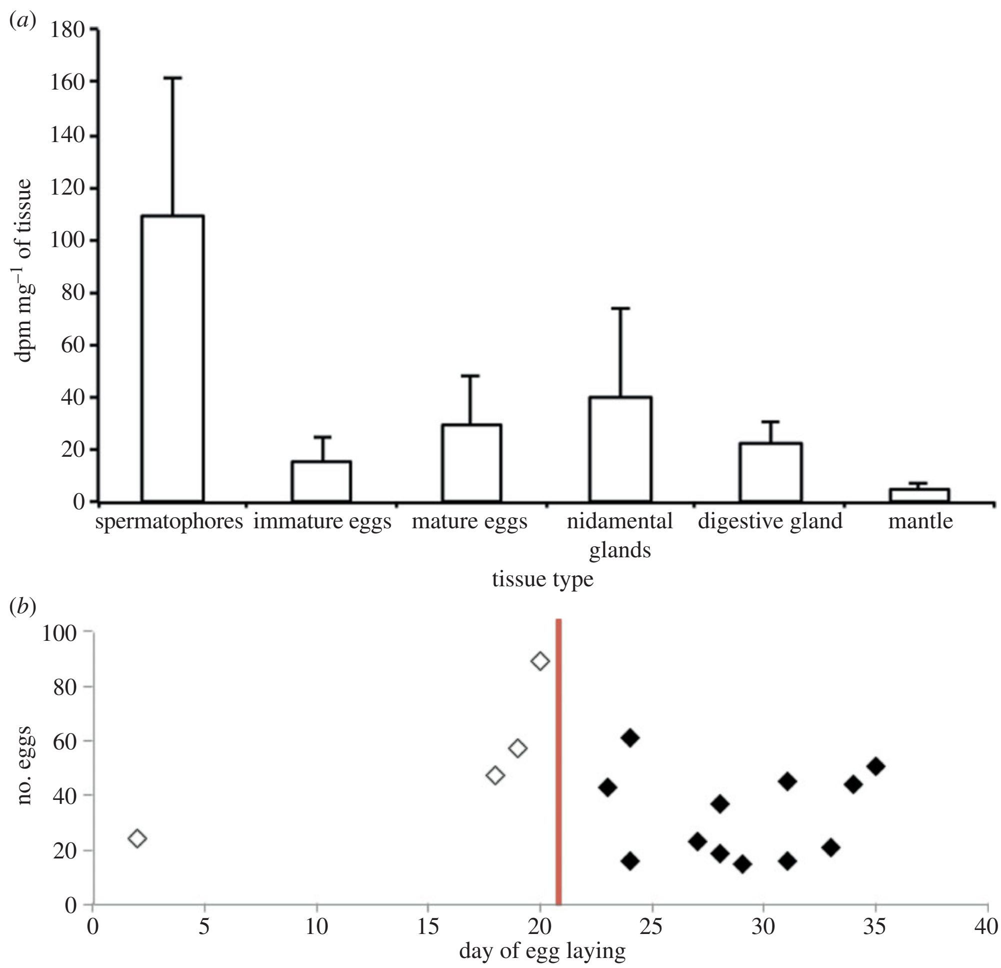

Figure 2. (*a*) Average ^{14}C radioactivity of spermatophores from radiolabelled males, and tissues from mated females. All tissues aside from the mantle have significantly higher radioactivity than background levels. Bars indicate standard errors. (*b*) Egg clutches laid by female squid after mating with two males on day 0. White markers indicate fertilized clutches. Black markers indicate unfertilized clutches. The line at 21 days shows the estimated time limit for female sperm storage. (Online version in colour.)

fertilized and unfertilized clutches (mean $\pm$ s.e. eggs per female: fertilized $= 54.25 \pm 2.25$, unfertilized $= 39.23 \pm 5.39$, independent *t*-test: $t = 1.984$, $p = 0.159$).

4. Discussion

This species represents the first documentation of spermatophore consumption in a cephalopod. Female southern bottletail squid consistently consume a portion of the spermatophores passed to them during copulation and use the nutrients received for somatic maintenance and oocyte development.

Unlike previously reported species exhibiting gamete consumption [5–10], the sperm of male southern bottletail squid do not enter the female reproductive tract. All spermatangia (and their accompanying sperm) are retained in the external buccal cavity for approximately three weeks, after which time they appear to no longer persist for egg fertilization (figure 2*b*). There are four possible mechanisms that contribute to the loss of stored spermatangia in females. First, the act of spermatophore consumption itself, possibly as a means of cryptic female choice; second, possible removal by rival males during copulation [13,19]; third, the absence of any internal seminal receptacle leaves spermatangia open to degradation through exposure to the environment; and fourth, given the site of storage is around the mouth of the female, the handling and consuming of struggling food items may inadvertently lead to the rupturing and loss of spermatangia.

The combination of gamete consumption and short-term external sperm storage has the potential to influence the copulatory strategies of both sexes in this species. Not only must females mate repeatedly during the six-month long breeding season to ensure viable sperm stocks, but males may also lose their reproductive investments should females fail to lay eggs soon after copulation. The latter is particularly relevant early in the breeding season, where males mate with females that are yet to produce mature oocytes (B. Wegener 2013, personal observation). Although such short-term sperm storage may cause males to miss fertilization opportunities, they may be compensated with alternative fitness benefits. This has been demonstrated, for example, in female cuttlefish, *Sepia officinalis*, which prefer to mate with males that have most recently mated [20]. Such possibilities in *S. austrinum* warrant further investigation.

Female spermatophore consumption is a consistent reproductive cost for male bottletail squid, with all mated females in the study exhibiting this behaviour (see the electronic supplementary material, movie S1). To counter this, males may use several strategies. First, males may be able to minimize spermatangia consumption through strategic placement in the female's buccal cavity. Behavioural observations indicate females struggle to remove spermatangia positioned towards the base of their beak. This was also supported during buccal cavity dissections, with numerous spermatophores typically found lining the base of the beak. Second, males may exhibit strategic mate choice by targeting those females closest to egg laying condition [21]. Such a strategy could further maximize the likelihood of successful egg fertilization before the loss of sperm stocks. Last, similar to many other species [22–24], male ejaculates may possess some manipulative compounds that stimulate female reproduction. Although we found no evidence for this in our study, with the majority of females failing to lay fertilized clutches before the depletion of their sperm stores (figure 2*b*), the laboratory environment may have been suboptimal for egg laying.

In conclusion, we have demonstrated ejaculate consumption in a cephalopod with external sperm storage. The discovery of this behaviour may have important implications for the reproductive strategies of both sexes in this species. Although females may use these traits to exert control over the paternity of their offspring, examining just how males manipulate such decisions could provide valuable insights into how sexual conflict shapes cephalopod reproduction.

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