Male copulatory behavior interrupts Japanese flying squid *Todarodes pacificus* female spawning activity

Running page head: Female spawning interruption by male squid

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Abstract

Batch spawning, intermittent spawning and multiple spawning represent common reproductive strategies among cephalopods. These flexible spawning strategies are also common in fishes, and are thought to be a female trait that is programmed depending on environmental parameters. The ommastrephid squid *Todarodes pacificus*, being a terminal spawner, is considered to have a single spawning event, extruding one large egg mass and dying soon thereafter. Females that are interrupted by males exhibiting mating behavior, while extruding the egg mass, instead spawn multiple egg masses over the course of 2–3 days instead of dying soon after spawning the first egg mass. We demonstrate that male mating behavior causes "forced" intermittent spawning by females (i.e., more than one spawning event). We hypothesize that in *T. pacificus*, some males use this strategy to mate with females unable to repel advances while spawning, and thus providing the male with the opportunity to contribute sperm and enhance gene flow.

Keywords: batch spawning, spawning interruption, multiple paternity, egg mass, oceanic squid

INTRODUCTION

Coleoid cephalopods are considered semelparous (i.e., no gonadal resting phase) (Mangold 1987), with the exception of the recently reported iteroparous vampire squid (Hoving et al. 2015). Although the spawning pattern of cephalopods is monocyclic (single spawning season), semelparity occurs in species that spawn eggs in single or multiple events, with their reproductive strategies being considered very flexible (Pecl 2001). The terms "batch spawning," "intermittent spawning," and "multiple spawning" are common in studies of cephalopod reproductive biology, particularly among species of the family Ommastrephidae, with these

terms being used interchangeably (Rocha et al. 2001). This family contains a few species that spawn (lay egg masses) intermittently, with no somatic growth between spawning events (Nigmatullin & Laptikhovsky 1994, Nigmatullin 2011). For instance, *Todarodes pacificus*, an ommastrephid squid, is a semelparous cephalopod mollusk that lays eggs by embedding oocytes inside a large egg mass (Sakurai et al. 2013). Being an intermittent terminal spawner, the female's feeding ceases prior to spawning (Bower & Sakurai 1996) in order to allocate all available energy to spawning, during which the female undergoes strenuous muscular contractions, involving the mantle, arms, and tentacles, while extruding the egg mass (Hamabe 1962).

The multiple spawning events of semelparous species are assumed to be a programmed reproductive strategy (Rocha et al. 2001); however, the reason why all spawning events by these individuals occur in the final days or weeks of their life cycle remains unknown (lifespan ≈ 1 y) (Rocha et al. 2001). For *T. pacificus*, the reported residual fecundity and potential fecundity are >150,000 and 320,000–470,000 (Soeda 1956) respectively. Thus, it is possible that the multiple spawning events (in the absence of somatic growth) by this reported terminal spawner are the result of unexpected interruptions that have remained unverified due to the absence of direct observation. Spawning by ommastrephid squid has never been observed in nature, as they spawn at depths (thermocline/pycnocline) that are not easily accessible to humans (Sakurai et al. 2000). Here, we investigated the reproductive strategy of female *T. pacificus* by facilitating and observing the interactions of mature males and females in a laboratory setting.

MATERIALS AND METHODS

The experiment was conducted from September to October 2015 in a large experimental tank (10 m [length] × 5 m [width] × 6 m [height]; volume = 300 m^3) located in the Hakodate

59 Research Centre for Fisheries and Oceans (HRCFO), Japan. The experimental tank was designed
60 according to previous experiments performed in our lab (Puneeta et al. 2015). Maximum water
61 turnover was kept very low (5 t h^{-1}) to prevent damage to spawned egg masses.
62 Mature adult squid belonging to the autumn cohort (Goto 2002, Yamamoto et al. 2002)
63 were collected from the coastal waters of southern Hokkaido, Japan, via trap nets and hand
64 jigging, onboard the T/S *Oshoro Maru*, during September 2015. Live squid were transported to
65 HRFCO and housed in a small tank (4 m [diameter] × 1 m [height]; volume = 10 m^3) until the
66 start of the experiment. Squid were fed a diet of frozen Pacific saury (*Cololabis saira*) every day
67 at 09:00 h. Nine copulated females [Mantle length (ML) range 20 - 28 cm, mean 24 cm) and two
68 mature males(ML: 19 &24 cm) were measured, and introduced to the large experimental tank for
69 the experiment. Each individual was also tagged with a labelled plastic color-coded ribbon tag on
70 one of its fins. Daily feeding was continued in the large experimental tank as aforementioned.
71 The spawning and mating behavior of squid were video-recorded by using fixed and
72 handheld Sony HDR-CX590V handycams (Sony, Tokyo, Japan). All video footages were
73 annotated, reviewed, and analyzed. Selected sequences from the videos (30 frames s^{-1}) were
74 captured with Adobe Premiere and exported as frames into ImageJ (http://imagej.nih.gov/ij/) to
75 observe the details of spawning behavior.
76
77 **RESULTS**
78 We obtained 21 egg masses from 9 females of varying sizes (diameter range: 15 to 120
79 cm, 59.3 ± 23 mean ± SD, Fig. 1). Feeding of squid was normal before and post-spawning. The
80 presence of more egg masses than female individuals provided evidence of multiple spawning
81 events. A complete spawning event by *T. pacificus* requires at least 7 min, and results in large

egg masses, greater or equal to 60 cm in diameter (Puneeta et al. 2015). During one spawning event, a male (ML: 24 cm) was found to first embrace a female (ML: 24 cm) and then start mating (Fig.2, for movie see supplement information). This behavior prevented the female from extruding the egg mass completely and she produced a single egg mass of just 20 cm in diameter. The next day, the same female spawned again, producing a second, much larger egg mass (diameter = 50 cm), and subsequently died. Thus, if male mating behavior interrupts female egg mass production, she may produce more than one egg mass before completing her life cycle.

The males in the school preferred to mate with females that were preparing for spawning. Out of 16 mating attempts observed, only four mating attempts occurred when females were swimming in the school, and the remaining attempts were made by males when females departed from the school and moved deeper into the water column for spawning. The female would be in a static position, with the posterior mantle inclined in a vertical posture, and flashing chromatophores, indicating the commencement of spawning. All mating attempts observed were complete, lasting for 10–18 s. All mating events were performed with the male in a parallel position (Sakurai et al. 2013) with the female and likely involved spermatophore transfer during all events. It is worth noting that the female did not demonstrate mate choice, which might have been evident if males approaching for mating had been rejected.

DISCUSSION

The production of two or more egg masses during a single reproductive cycle is termed intermittent spawning. This spawning strategy has many advantages (e.g., reducing predation pressure and allowing for wider dispersion) (Vijai et al. 2014, Vijai et al. 2015); however, females are thought to control this strategy by delegating energy input to reproduction (Ramirez Llodra 2002). Maturation and ovulation in animals may be induced and controlled by exogenous

hormones (Hong & Zhang 2003); however, in nature, reproductive strategies are considered unique animal characteristics. Multiple mating and resulting multiple paternity is common in cephalopods (Boyle & Rodhouse 2005), but the influence of competition for mates affecting the spawning strategy as a whole is unknown.

Spawning grounds are usually areas characterized by optimum environmental conditions and minimal predator density. In natural squid spawning areas, males and females coexist (Tafur et al. 2001). In fact, female loliginid squid pair with males while spawning (Hanlon et al. 2004). In general, male cephalopods compete intensely for mates, with females rejecting up to 70% male mating attempts in some species (e.g., *Sepia apama*) (Hanlon et al. 2005). In *T. pacificus*, during copulation, males place their spermatophores on the buccal membrane of females (Okutani 1983). Spermatozoa are subsequently stored in the female's seminal receptacles for several weeks until spawning. Fertilization only occurs at spawning, with a single female copulating multiple times before spawning (Puneeta et al. 2015); thus, the embryos from a single spawning event may have multiple fathers. To extrude the egg mass, females utilize the funnel, normally used for locomotion (jet propulsion). This results in immobility while spawning, making the female more vulnerable to males. Thus, weaker males in a cohort may attempt to copulate with females at this point, enhancing their gene flow and fitness through sperm competition. In essence, when females are in the spawning process (about to spawn or in the process of spawning) is a crucial factor in mate choice by males.

Our observation indicates that in *T. pacificus*, males may actively interrupt female spawning events. This behavior resulted in forced "intermittent spawning," which is normally considered a strategic spawning mode. We were unable to determine whether the male was aware that the female was spawning or whether it was simply exploiting the female's vulnerable

position. In nature, the interruption of spawning may also arise for other reasons such as predator pressure. Our results support theories of stress-induced multiple spawning, showing that flexible reproductive strategies by *T. pacificus* are highly adaptable based on immediate circumstances, especially while considering the absence of somatic growth between their spawning events.

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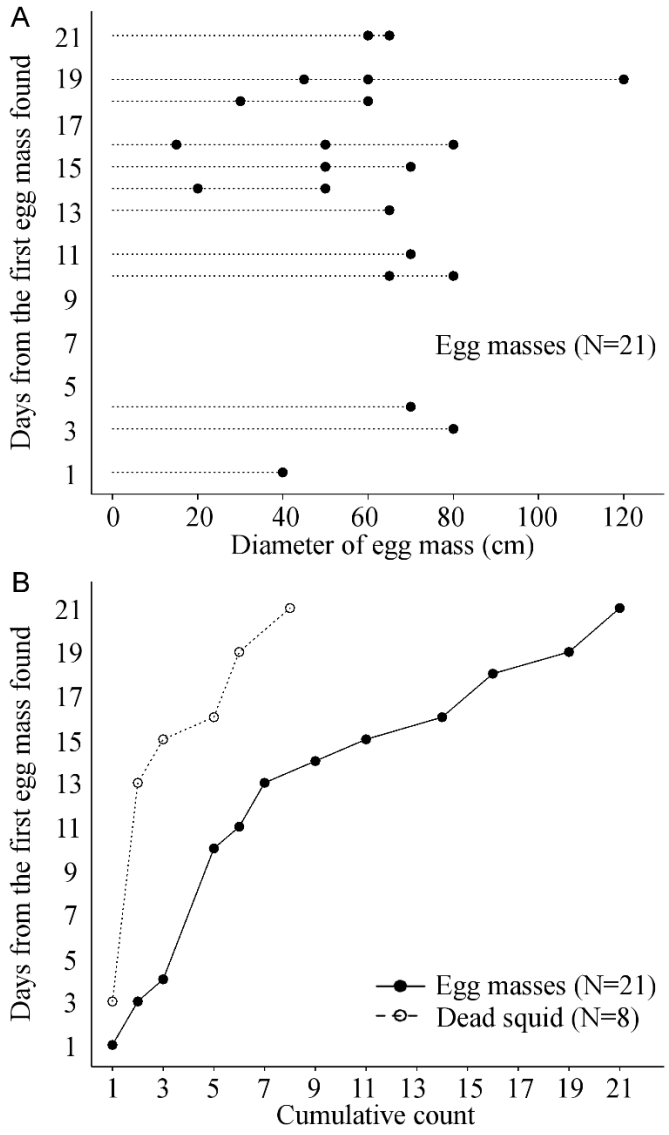
196 **Figures**



197

198 Fig. 1. Post-spawning summary of the spawning events of the ommastrephid squid, *Todarodes*

199 *pacificus*, inside the experimental tank. (A) Size of egg masses and (B) Number of egg masses

200 and dead post-spawn females (of the total 9 females the last one was manually removed).

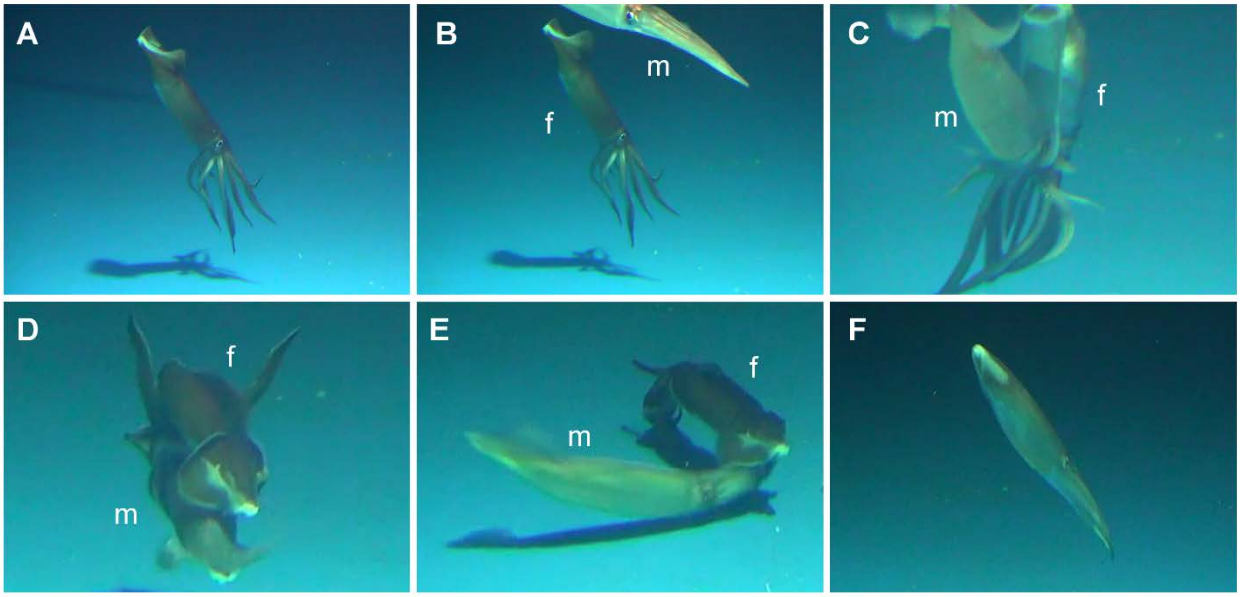


Fig. 2. Male copulatory behavior interrupts female spawning activity. (A) A spawning female. The egg mass is completely transparent (visible only with light from a flashlight); (B) male approaching; (C) male grabbing the female from ventral side; (D) mating in “male parallel” position; (E) male withdraws after copulation; (F) spawning interrupted female departs.m: male; f: female.(See the electronic supplement for video clip).