1   **Complex adaptive traits between mating behaviour and post-copulatory sperm behaviour in squids**

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14   **Abstract**

15   Emergence of male dimorphism within a species is the evolutionary process of disruptive selection. In

16   squids, two types of male mating behaviour, known as alternative reproductive tactics (ARTs), are

17   causally associated with adult body size. Males inseminate promiscuously with the same females; large

18   "consort" males internally, and small "sneaker" males externally. Previously we found that in

19   *Heterololigo bleekeri*, sneaker (but not consort) spermatozoa are able to swarm by sensing self-emitted

20   CO_2. This suggests that a swarming trait might have arisen in sneakers as a "sperm cooperation" strategy

21   among sibling sperm in order to compete with consort males, or as a consequence of adaptation to

22   external fertilization. To address these possibilities, we examined six species where three patterns of

23   insemination are present, namely, only internal, only external, or both ARTs. In three species that employ

24   both ARTs (*H. bleekeri*, *Loligo reynaudii* and *Uroteuthis edulis*), sneaker spermatozoa always exhibited

25   self-swarming capacity. In *Idiosepius paradoxus* and *Todarodes pacificus*, which use only external

26   insemination, spermatozoa formed a swarm. However, in *Euprymna morsei,* which use only internal

27   insemination, sperm were unable to swarm. These results suggest that the self-swarming trait is likely to

28   be linked to the mode of insemination rather than the alternative strategy used by sneaker males. Thus we

29   propose a new hypothesis in which cooperative sperm behaviour has evolved not only through kin

30   selection against sperm competition risks, but also through adaptation to the insemination/fertilization

31   environment.

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33   **Keywords:** Alternative reproductive tactics, sperm trait, sperm competition

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36   **Introduction**

In sexually reproducing organisms, a diverse array of mating or post-mating strategies has evolved through sexual selection or post-copulatory sexual selection, such as sperm competition and cryptic female choice, Male dimorphism may be accounted for as evolution favouring two discontinuous niches for mating opportunities arising from intra-sexual conflict (Danforth and Desjardins 1999). Male dimorphism is usually linked tightly to ARTs, in which animals with certain phenotypes use a mating strategy that differs from the predominant strategy in order to maximize reproductive success, or to overcome a reproductive disadvantage (Emlen 1997). In cephalopods alternative reproductive strategies by males have long been recognized (Drew 1911b). For example, two distinguishable insemination modes are present in *Loligo pealei:* in one, males transfer the sperm ampulla (spermatophore) to the inner side of the outer buccal membrane, where the seminal receptacle is located (external insemination); in the other, the spermatophore is affixed near the end of the oviduct (internal insemination) (Drew 1911a). Consort males form temporary pairs with females on the spawning ground and spawning follows the pairing. Similar divergent behaviours, with males exhibiting two distinctly different modes of insemination within the same species, have been found in other Loliginidae species, including *Heterololigo bleekeri* (Iwata et al. 2005) and *Loligo reynaudii* (Hanlon et al. 2002b). In cuttlefish, body-size related ARTs are known in *Sepia apama:* large males fight with other males and guard females, and small males mimic females and attempt sneaking copulation (Hall and Hanlon 2002). This female-mimic tactic by small males increases the chance of mating (Hanlon et al. 2005). However, the mechanisms determining these different tactics and any fitness advantages remain largely unknown.

This alternative choice of male mating behaviour could depend on the individual condition of each male [for review see (Gross 1996)]. In the oval squid, *Sepioteuthis lessoniana*, males change mating tactics depending on the relative size-difference between sexes (Rocha et al. 2001; Wada et al. 2005). Such plasticity in mating tactics has been noted in *Sepioteuthis lessoniana,* where the same individuals exhibit both parallel and upturned male mating behaviours in terms of orientation to the female (Wada et al. 2005). In *H. bleekeri*, there are ARTs where larger males attempt internal, and smaller males external insemination, as seen in *L. pealei.* In *H. bleekeri*, however, the selected tactic is primarily determined through ontogeny, as sperm traits are constrained by the mode of insemination, i.e. smaller males attempt sneak copulation and deposit the spermatophore externally, and larger males attempt male-parallel copulation and deposit the spermatophore internally (Iwata and Sakurai 2007; Iwata et al. 2011).

An extreme difference in ejaculation between sneakers and consorts is the self-swarming observed only in sneaker sperm of *H. bleekeri* (Hirohashi et al. 2013; Hirohashi and Iwata 2013). Self-swarming is triggered by chemotaxis stimulated by respiratory CO_2 emitted by sibling sperm. Self-swarming behavior by sneaker sperm could be regarded as a form of sperm cooperation because sibling sperm forming a

70 cluster has been well recognized in some species as an evolutionary consequence of kin selection under

71 intense competitive conditions (Moore et al. 2002). In general, sneaker individuals have a higher sperm

72 competition risk because co-existing large consort males have favorable mating opportunities of timing

73 and position. Thus, the ARTs system itself could be a driving force in producing polymorphic sperm

74 traits as a result of developmental plasticity. Alternatively, ARTs in loliginid squid may be a response to

75 physical (and chemical) differences in the insemination/fertilization environment, and these differences

76 could drive adaptive evolution in sperm. Such ARTs are known only in the loliginid family. On the other

77 hand, having a seminal receptacle (SR) is common in various cephalopod species. Externally deposited

78 sperm are thereafter transferred to and stored in the seminal receptacle (SR). Therefore, if the

79 self-swarming behaviour of loliginid sneaker sperm has resulted from adaptation to an external sperm

80 storage environment, then the sperm of other cephalopod species may possibly exhibit similar behavior.

81 To examine this possibility we investigated whether the self-swarming trait found in the sneaker sperm of

82 *H. bleekeri* is also a characteristic of other squid species with ARTs, and of species that employ only

83 external insemination and do not have ARTs.

84

85 **Materials and methods**

86 Specimens of *Heterololigo bleekeri* were obtained from fisheries at three locations: Sagami Bay, the

87 Tsugaru Strait, and the Oki Islands (Fig. 1a). Spermatophore sacs with developed spermatophores were

88 removed from mature male squid immediately after dissection and kept in a moist chamber on ice.

89 *Uroteuthis edulis forma kensaki* (Takemoto and Yamashita 2012) were obtained from fisheries in the Oki

90 Islands. *Idiosepius paradoxus* and *Todarodes pacificus* were collected in Chita Bay and Sagami Bay,

91 respectively. *Euprymna morsei* was obtained from the fishery at Sakai Port. *Loligo reynaudii* was the only

92 species collected outside Japan, off Port Elizabeth in South Africa. Sites of sperm transfer with different

93 species are illustrated in Fig. 1b. *H. bleekeri* and *L. reynaudii* are known to have ARTs (Hanlon et al.

94 2002a; Iwata and Sakurai 2007). Depending on the morphology of ejaculated spermatangium, each

95 individual was distinguished as consort or sneaker (Iwata et al. 2015). As ARTs for *U. edulis* were not

96 known, we carried out anatomical investigation of collected male and female individuals (Fig. 1c). Male

97 *U. edulis* exhibited clear dimorphism in spermatophore morphology and sperm flagellar length, and the

98 spermatozoa stored in the seminal receptacle were exclusively sneaker type. In *I. paradoxus* and *T.*

99 *pacificus,* males only inseminate externally, which is similar to sneaker male mating tactics (Ikeda et al.

100 1993; Sato et al. 2010). The anatomy of *E. morsei* suggested that males inseminate exclusively inside the

101 mantle cavity, as reported for *E. tasmanica* (Norman and Lu 1997).

102   Three different assays were tested to verify the sperm-swarming trait as Hirohashi et al. (2013)

103 described previously. First, we stimulated a spermatophoric reaction in a seawater-filled petri dish. The

104 manner of sperm emission was observed under a microscope. Second, ejaculated spermatozoa were

105 suspended in seawater and taken up into a 5 μl capillary tube. *I. paradoxus* and *E. morsei* were not tested

106 in this assay because very few spermatozoa were obtained. Third, if specimens were available in the

107 laboratory, a bubble of CO_2 gas was introduced into the sperm suspension using a micromanipulator.

108 Carbonated seawater was used only if specimens were obtained during the fieldwork (*L. reynaudii* and *E.*

109 *morsei*). The first assay reproduced a more physiological condition than would occur during copulation.

110 The second and the third assays were experimental and tested for chemotaxis , particularly toward CO_2 in

111 the third assay. For each species, assays were performed at least three times using different male

112 individuals.

113

114 **Results**

115 We first tested whether the previously reported sperm behaviour phenotype—self-swarming of

116 spermatozoa in *H. bleekeri*—would be exhibited consistently and globally in sneaker male individuals.

117 Evaluation using three assays (see Materials and Methods) (Hirohashi et al. 2013) showed that sneaker

118 spermatozoa from all three locations formed a swarm (Table 1), supporting our previous hypothesis that

119 this ejaculate trait is tightly coupled with the ontogeny of sneaker males.

120   Next we investigated whether sneaker males of other squid species, *L. reynaudii* (Hanlon et al.

121 2002a) and *Uroteuthis edulis,* both of which show male dimorphism in terms of relative length of their

122 mantle, exhibit a similar result. In *L. reynaudii,* male dimorphism is correlated with alternative

123 insemination sites; however, evidence for ARTs in *U. edulis* has not been established but is presumed,

124 based on the anatomical observations described above. As with *H. bleekeri*, self-swarming occurred only

125 in the sperm of sneaker males (Iwata et al. 2015) of these species.

126   We then tested *T. pacificus* and *I. paradoxus* where only external insemination is present (Sato et

127 al. 2010). *T. pacificus* sperm exhibited self-swarming in the vicinity of the ejaculation duct when

128 ejaculated from the spermatophore. Unexpectedly, they failed to re-swarm after being suspended and

129 loaded into a capillary tube. However, self-swarming was restored when a CO_2 bubble was introduced

130 into the tube. *I. paradoxus* sperm also exhibited self-swarming immediately after release from the

131 spermatophore, but once sperm were diluted, re-swarming did not occur, regardless of attempts to

132 stimulate re-swarming. These results suggest that the mechanism underlying self-swarming in *I.*

133 *paradoxus* differs from the other species.

134   Finally, we tested *E. morsei*. In this species, spermatangia are placed exclusively on the right side

135 of the inner wall of the female's mantle (but not in the buccal cavity) (Fig. 1d), suggesting only internal

136 insemination. Sperm ejaculated from the spermatophore were motile and no self-swarming was observed

137 in the vicinity of the duct. When a CO_2 bubble was introduced, neither self-swarming nor directional

138 movement to the bubble was observed. These results suggest that it is unlikely that the self-swarming trait

139 is present in *E. morsei* sperm.

140

141 **Discussion**

142 We found that *H. bleekeri* sneaker males produce sperm that exhibit self-swarming in response to

143 respiratory CO_2 (Hirohashi et al. 2013). Theoretically, sneaker males should produce better quality sperm

144 than that of consort males because sneaker males are usually disadvantaged by the presence of the

145 dominant sperm of consort males that have superior access to eggs (Parker 1990). Empirical evidence of

146 better quality sneaker sperm was found in the fish *Xiphophorus nigrensis:* sperm from sneaker males

147 swim faster (Smith and Ryan 2010). In the common wood mouse (*Apodemus sylvaticu),* sperm form

148 sibling aggregates to swim faster than those that do not form aggregates (Moore et al. 2002). Such

149 aggregation is regarded as cooperative behaviour. Taking into account these examples of ejaculate

150 investment by parasitic males, self-swarming by sneaker sperm observed in squid could be a consequence

151 of sperm competition, particularly against consort males. However, in *H. bleekeri,* swimming speed of the

152 two types of sperm was similar, and sperm in the swarm did not show facilitated movement. The rationale

153 for this behaviour is still unclear; a logical next step would entail direct observation to determine whether

154 sneaker and consort sperm compete for fertilization of the same ova.

155   Another point to consider in the evolution of ejaculate traits in squid is the post-copulation processes

156 leading to successful fertilization. Squid ARTs offer a unique situation, because, despite a wide variety of

157 ARTs recognized throughout animal phyla, the occurrence of more than one form of male mating

158 behavior, with different targets for insemination, is rare. In loliginid squids, the spermatozoa of males

159 using the internal insemination mode, encounter eggs immediately after ejaculation, presumably within

160 the female's mantle cavity, whereas with external insemination, the ejaculated sperm are translocated and

161 stored in the seminal receptacle (SR) until the eggs are deposited. Although the duration of sperm storage

162 in the SR remains unknown in these species, females of many cephalopod species store sperm, sometimes

163 for several months (Mangold 1987). In general, sperm storage in the SR is an integral component of the

164 female's reproductive strategy because it secures fertilization, despite the possible absence of males at the

165 time of egg-laying. In squids, sperm storage is also advantageous for sneaker males, enabling them to

166 avoid competition from other males in the vicinity of the spawning grounds. Notably, the female SR is

167 present not only in species with ARTs, but also in species employing external insemination only, such as

168 *Todarodes pacificus* and *Idiosepius paradoxus* (Ikeda et al. 1993; Sato et al. 2010). Furthermore, there are

squid species (e.g. *Euprymna tasmanica*) that store sperm internally in the female for months. This location can exhibit differentiation in sepiolid squids (e.g. *Sepietta, Euprymna and Sepiola*) as a complex enlargement of the distal oviduct (Hoving et al. 2008). Future studies should carefully address whether the swarming trait of sperm is associated with the mode of sperm storage or of the insemination environment.

In loliginid squid, we speculate that, because females have a well-developed SR specific to sneaker sperm, sneaker male tactics could be an ancient form of insemination, and consort males acquired internal insemination and lost the sperm-swarming trait, which is unlikely to be the case for other species with ART (Fig.1c). Self-swarming may be an adaptive trait in response to the very different environments, i.e. within the mantle cavity or around the buccal cavity, resulting in a co-evolution of sperm traits, possibly by cryptic female choice, i.e. the female SRs preferentially store sperm with higher swarming capacity (Naud et al. 2005). In contrast, no differentiation of tissue is apparent at the site of sperm placement by dominant consort males.

Our current study clearly showed that the self-swarming trait does not differ within a species, samples of which were collected from three distinctly different geographical spawning areas, or between closely related species that employ ARTs. However, the study found a relationship between the male's insemination mode and the ejaculate's swimming behaviour. To understand more fully the physiological role(s) of the observed swarming behaviour by sneaker sperm, we need to understand the post-copulation processes involved in successful fertilization of the eggs, which could be achieved by measuring the step-by-step success in copulation, sperm storage in the SR, and fertilization (paternity) under experimental conditions. In species with ARTs, the choice of ART by males is often more responsive to mating conditions, whereas sperm traits are endowed during gametogenesis. In this context, males of *H. bleekeri* may express behavioral plasticity independent of intrasexual competition.

The current study offers new insight into ARTs research, particularly regarding complex adaptive traits between mating behaviour and post-copulation sperm behaviour. The study confirms the very complex reproductive strategy in squid, and the importance of understanding this complexity, particularly when fishing practices may target mating and spawning animals, often selectively.

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273 **Figure legend**

274 **Fig. 1** (*a*) Locations and time period (month) for each squid species collected off the coasts of Japan. *Hb*,

275 *H. bleekeri*; *Em*, *E. morsei*; *Ue*, *U. edulis*; *Tp*, *T. pacificus*; *Ip*, *I. paradoxus*. (*b*) Arrows indicate two

276 distinct insemination sites used by different squid species; female reproductive organs (ovary and

277 oviduct) illustrated in red. (*c, d*) Discharged spermatophores (spermatangia, indicated by *cyan arrows*)

278 anchored on the inner buccal membrane located externally on the body of the female squid, *U. edulis* (*c*)

279 and inside the mantle cavity, *E. morsei* (*d*). Bar, 30 mm; SR, seminal receptacle; *yellow arrow*, buccal

280 membrane.

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282 **Table 1** Sperm-swarming trait and male insemination modes in squids

species	Location collected	Male size dimorphism	Insemination sites	Upon spermatophoric reaction	Inside a capillary tube	Toward a CO$_2$ bubble
Heterololigo breekeri	(Tsugaru Strait, JPN)	yes (cs/sn)	int (cs)	no (cs)	no (cs)	no (cs)
			ext (sn)	yes (sn)	yes (sn)	yes (sn)
	(Sagami Bay, JPN)	yes (cs/sn)	int (cs)	no (cs)	no (cs)	no (cs)
			ext (sn)	yes (sn)	yes (sn)	yes (sn)
	(off Oki Islands, JPN)	yes (cs/sn)	int (cs)	no (cs)	no (cs)	no (cs)
			ext (sn)	yes (sn)	yes (sn)	yes (sn)
Loligo reynaudii	(off Pt. Elizabeth, SA)	yes (cs/sn)	int (cs)	no (cs)	no (cs)	no (cs)
			ext (sn)	yes (sn)	yes (sn)	yes (sn)
Uroteuthis edulis	(off Oki Islands, JPN)	yes (cs/sn)	ND (cs)	no (cs)	no (cs)	no (cs)
			ext (sn)	yes (sn)	yes (sn)	yes (sn)
Idiosepius paradoxus	(Chita Bay, JPN)	no	ext	yes	ND	no
Todarodes pacificus	(Sagami Bay, JPN)	no	ext	yes	no	yes
Euprymna morsei	(off Sakai-Pt., JPN)	no	int	no	ND	no

283 cs: consort males, sn: sneaker males, int: internal site, ext: external site, ND: not determined

284 Male type, i.e., sneaker or consort, was determined by morphology of spermatangium (Iwata et al. 2005)

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