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Threshold dimorphism in ejaculate characteristics in the squid *Loligo bleekeri*

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Running head: Dimorphic ejaculate characteristics in squid

Male dimorphism has been thought to correlate with alternative reproductive behaviors. Alternative reproductive behaviors promote asymmetry in sperm competition, and the differences in fertilization success could promote adaptations in ejaculate characteristics in relation to each reproductive behavior. Here, using allometric analysis, we show that there is clear ejaculate dimorphism in males of the squid *Loligo bleekeri*, a species with
body-size related alternative mating behaviors. A morphological switch point was detected in internal characters: larger individuals made discontinuously longer spermatophores than smaller individuals, although any switch point was not detected in external characters (fin length, fin width, head width, mantle width, tentacle length and hectocotylus length) except bimodal body size. This clear internal switch point could be an adaptation to the characteristic alternative mating behaviors of loliginid squid, in which males use different mating tactics to pass spermatophores to different sperm storage sites in and on the females. This study reports on clear dimorphism in ejaculate characteristics in cephalopods for the first time, to our knowledge. Our results indicated that alternative reproductive behaviors can result in morphological adjustment in internal characters.

KEY WORDS: male dimorphism, sperm competition, alternative reproductive behavior, squid, *Loligo bleekeri*
INTRODUCTION

In many species, the expression of secondary sexual traits of individuals varies within a sex (Andersson 1994). Game theory suggests that each individual will choose a phenotype that achieves the highest fitness, depending on its competitive ability or social status (Gross 1996). Such a conditional strategy will have a phenotypic switch point, at which the fitnesses of the two phenotypes are equal (Gross 1996). The most common conditional reproductive strategy has two alternative reproductive behaviors that depend on body size (“pair mating” and “sneaking”), and such alternative reproductive behaviors promote the evolution of male dimorphism (Eberhard 1982, Gross 1996, Taborsky 1998).

Males in some species are dimorphic at maturation, with large males having fully developed secondary sexual characters and small males having less-developed secondary sexual characters (e.g. Eberhard and Gutiérrez 1991). Male dimorphism would be favored by heterogeneous selection for discrete alternative mating behaviors. Then, finding a morphological switch point is important for understanding status-dependent fitness functions of alternative mating behaviors.

Alternative mating behaviors result in not only external male dimorphism but also internal-morphological and physiological adaptations. Theoretical models predict that
sneaker males with constantly high sperm competition risk will expend reproductive effort on sperm expenditure for the disadvantage in behavioral competition (Parker 1990a, 1990b, Parker et al. 1997). Many studies in various species have shown that sperm competition risk affects sperm investment patterns such as ejaculate characters (Snook 2005).

Studies of the ejaculate strategies associated with alternative mating behaviors in both external-fertilization and internal-fertilization animals support the prediction of sperm competition theory (e.g. Gage & Barnard 1996, Leach & Montgomerie 2000, Evans et al. 2003, Nicholls et al. 2001). In those animals examined, the ejaculate- and sperm-storage sites which are used for each alternative mating behavior are not perfectly separated, even though sperm release timings and/or mating orders differ among males. Female of loliginid squid, however, have two distinct sperm storage sites (the seminal receptacle near the mouth and the opening of the oviduct within the mantle cavity), corresponding to alternative mating behaviors (Hanlon & Messenger 1996). Loliginid squids make dense spawning aggregations on coastal spawning grounds, and males pair temporarily with females and mate with them (Hanlon & Messenger 1996). These spawning aggregations usually tend to have more males than females, and some males copulate by sneaking (Hanlon et al. 1997, 2002). The mating behaviors that males use correlate with their body
size: large males pair with females and copulate in the male-parallel position by attaching spermatophores inside the female’s oviduct in the mantle cavity, and small sneaker males mate in the head-to-head position by attaching spermatophores near the mouth of females that have already paired with other males (Hanlon et al. 1997, 2002). Sneaker males fertilize fewer eggs than paired males do (Iwata et al. 2005). The fact suggests that sperm stored near the oviduct have an advantage, perhaps because the eggs being spawned will meet sperm stored in the oviduct before the sperm stored in the seminal receptacle near the mouth. These size-dependent alternative mating behaviors and the differences in fertilization success could promote strategic ejaculation specializing in each sperm-passing site. Furthermore, loliginid males pass sperms as spermatophores, enclosing the spermatozoa within a hard shell, and males store several hundreds of spermatophores when they mature. Therefore, the ejaculate characters associated with male’s status can be measured more easily in loliginid squid than in other species that release sperm as milt. So, loliginid squid are ideally suited to study how ejaculate characters are related with alternative mating behaviors.

In the present study, we analyze the presence of a male morphological switch point for both external and internal characters in the squid Loligo bleekeri to determine if
alternative mating behaviors can result in male dimorphism.

MATERIALS AND METHODS

Sample collection

Samples of *Loligo bleekeri* were collected in inshore set nets in southern Hokkaido during the spawning season: February to March in 2004 and January to April in 2005. In 2004, we sampled 154 individuals randomly, and 72 individuals of those were males (size range: 136-341 mm). In 2005, we sampled 611 individuals from the commercially fished squid separated among three size classes: large (less than 18 individuals per 3 kg), medium (18 - 28 individuals per 3 kg) and small (more than 28 individuals per 3 kg). To cover all of the size range of *L. bleekeri*, we sampled 12 kg from large size class (162 males ranging 242 – 392 mm / 165 individuals ranging 242 – 392 mm), 8 kg from medium size class (76 males ranging 221 – 342 mm / 171 individuals ranging 204 – 342 mm) and 8 kg from small size class (92 males ranging 152 – 242 mm / 275 individuals ranging 126 – 252 mm). The body-size frequency distribution was calculated using the size distribution of the measured samples and catch weight of each size class on each sampling day. Of the 611 measured individuals, 330 individuals were males. The
maturity stages of all individuals in both years were determined according to the maturity stage indices described in Perez et al. (2002) for *Loligo plei*, and all individuals were fully mature.

For each male, we measured the mantle length, fin length, fin width, head width, mantle width, tentacle length and hectocotylus (4th arm specialized for transferring spermatophores to females) length. Furthermore, we selected ten spermatophores randomly from spermatophore storage organ for each male and measured their lengths using electronic slide calipers under a stereomicroscope. Mean and standard deviation of spermatophore length were calculated for each male. A total of 61 individuals in 2005 had only broken spermatophores in their spermatophore storage organ, so the spermatophore length was measured in the 269 other males in 2005.

Once spermatophores are passed on female’s body during copulation, sperm contents are ejaculated from spermatophore shell. The content attach on the female body as a “sperm mass”, in which milt are packed in thin pouch. For female samples, we examined the opening of oviduct and around seminal receptacle whether sperm mass were attached or not. If sperm mass were observed in the opening of oviduct and/or around seminal receptacle, we noted the shape of the sperm mass.
Data analysis

If more than one mode were observed in body size distribution, a normal mixture model was fitted to the distribution to describe a multi-modality of the body size (Fraley & Raftery 2002). The body size within each size range were assumed to be normally distributed, with density

$$f_i(x; \mu, \sigma) = \frac{1}{\sqrt{2\pi}\sigma^2} e^{-\frac{(x-\mu)^2}{2\sigma^2}}$$

and mean $\mu$ and variance $\sigma^2$. The overall size distribution is then a mixture of normal components,

$$f(x) = \sum_{i=1}^{k} \lambda_i f_i(x; \mu_i, \sigma_i)$$

one component for each size mode, the multipliers $\lambda_i$ representing the proportion that each size mode makes up of the entire distribution.

With the 72 and 330 males measured in 2004 and 2005 respectively, we tested for male dimorphism using the method of Eberhard and Gutiérrez (1991). First, we fit the partial regression equation to determine if the relationship between mantle length and each character was non-linear, which would indicate a potential of dimorphism. The equation is:

$$\ln Y = \alpha_0 + \alpha_1 \ln X + \alpha_2 \ln X^2 + \varepsilon \quad \text{(Model 1)}$$
where $Y$ is the length of each character, $X$ is the mantle length, $\alpha_i$ is the regression coefficient, and $\varepsilon$ is the random component assuming a normal distribution and homogenous variances. An $F$-test was conducted for the null hypothesis $H_0$: $\alpha_2 = 0$. If the coefficient $\alpha_2$ did not significantly differ from zero, we concluded that the characters showed no significant deviation from linearity and that further analysis was not necessary. If $\alpha_2$ significantly differed from zero, we concluded that the relationship was non-linear and the possible existence of dimorphism.

If the analysis indicated the relationship was non-linear, a second analysis was conducted to determine if there was a switch point, which was defined as the point where 1) the liner slope of mantle length ($X$) versus each character ($Y$) changed, and 2) the change in $Y$ was discontinuous. To test the discontinuity at a switch point, we fit the partial regression equation:

$$Y = \beta_0 + \beta_1 X + \beta_2 (X-X_0)D + \beta_3 D + \varepsilon \quad \text{(Model 2)}$$

in which $Y$ is the measurement of each character, $X$ is the mantle length, $X_0$ is the proposed switch point, $D = 0$ when $X < X_0$, $D = 1$ when $X_0 < X$, $\beta_i$ is the regression coefficient, and $\varepsilon$ is the random component assuming a normal distribution and homogenous variance. To determine the switch point giving the highest adjusted $R^2$ value, various $X_0$ were substituted.
in Model 2, with 5 mm steps through the observed mantle length (155 - 340 mm). Using the best-fitted switch point, a $T$ test was used to test the null hypothesis $H_0$: $\beta_3 = 0$. If the coefficient $\beta_3$ differed significantly from zero, we concluded that dimorphism occurred and it was discontinuous at the switch point.

If coefficient $\beta_3$ did not significantly differ from zero, a third analysis was conducted to test the change of liner slope of mantle length and each character at the switch point. We fit the partial regression equation:

$$ Y = \beta_0 + \beta_1 X + \beta_2 (X - X_0)D + \epsilon \quad \text{(Model 3)} $$

in which each term was defined as above, and the best switch point was determined in the same manner with Model 2. Using the best switch point in Model 3, a $T$ test was used to test the null hypothesis $H_0$: $\beta_2 = 0$. If coefficient $\beta_2$ differed significantly from zero, we concluded that dimorphism occurred with a significant difference in the slopes on either side of the switch point, but it was not discontinuous at the switch point.

RESULTS

In 2004, male mantle lengths ranged from 136 to 341 mm ($n = 72$) and varied more than those of females (164 - 258 mm, $n = 82$, Fig. 1-A). The body size distribution of
males and females were unimodal, but male samples contained more individuals of small size (Fig. 1-A). In 2005, male mantle lengths ranged from 152 to 392 mm (n = 330) and varied more than those of females (126 - 280 mm, n = 281, Fig. 1-B). The body size distribution of males was bimodal with two modes at mantle length 175 - 200 mm and 275 - 300 mm, and the distribution of females was unimodal (Fig. 1-B). We fitted a normal mixture model to the size distribution of males. The best fitting model was the mixture of two normal distributions, which include small ($\lambda = 0.204, \mu = 173.2, \sigma = 18.20$) and large one ($\lambda = 0.796, \mu = 270.6, \sigma = 29.57$). Cross point of two normal distributions located at mantle length 205 mm.

Table 1 shows the results of analyses for dimorphism in the seven characters examined in both years. In 2004, hectocotylus length and spermatophore length had significant values of $\alpha_2$ in Model 1. Using Model 2, spermatophore length showed significant discontinuities (reject $\beta_3 = 0$), which gave statistical evidence of threshold dimorphism (Fig. 2-A). Hectocotylus length was analyzed with Model 3, and the coefficient $\beta_2$ did not significantly differ from zero ($T$ test, coefficient = 0.12, $t = 1.26$, $p = 0.21$), which indicated that there was no dimorphism. In 2005, fin width and spermatophore length had significant values of $\alpha_2$ in Model 1. Using Model 2, spermatophore length
showed significant discontinuities (reject $\beta_3 = 0$), which gave statistical evidence of threshold dimorphism (Fig. 2-B). Fin width was analyzed with Model 3, and the coefficient $\beta_2$ did not significantly differ from zero ($T$ test, coefficient = -0.02, $t = -1.85$, $p = 0.07$), which indicated that there was no dimorphism. Thus, none of the external body characters (fin length, fin width, head width, mantle width, tentacle length and hectocotylus length) showed a significant deviation from linearity and spermatophore length showed dimorphism with a discontinuous switch point in both years. The best-fitting switch point for spermatophore length based on Model 2 occurred at mantle length 207 mm in 2004 and 221 mm in 2005: males smaller than this size had discontinuously shorter spermatophores than larger males (the estimated differences in spermatophore length between the longest short type and the shortest long type were 3.33 in 2004 and 3.22 mm in 2005, respectively).

In contrast, there was little variation of spermatophore length within each individual (average standard deviation of 10 spermatophore lengths = 0.439 in 2004 and 0.387 in 2005). Spermatophore lengths were positively related to mantle length both in the short type and in the long type in both years (in 2004, long type: $n = 65$, $Y = 0.022X + 9.755$, $R^2 = 0.480$, $F_{1,63} = 58.15$, $P < 0.001$, short type: $n = 7$, $Y = 0.029X + 4.947$, $R^2 = 0.838$, $F_{1,5} = 25.91$, $P < 0.01$, Fig. 2-A; in 2005, long type: $n = 212$, $Y = 0.021X + 9.703$, $R^2 = 0.315$,
Morphological observations on each spermatophore type showed that sperm mass ejaculated from short-type spermatophores had a drop-like shape (Fig. 3-A), and those ejaculated from long-type spermatophores had a rope-like shape (Fig. 3-B). Observations of sperm mass attached on the different body parts of females showed that all of the sperm mass attaching around the seminal receptacle was drop-like shape (n = 39 females, Fig. 3-C). In contrast, all of the sperm mass attaching in the oviduct of females was rope-like shape (n = 62 females, Fig. 3-D).

DISCUSSION

Most of alternative mating tactics involve the dimorphism in secondary sexual characters (Andersson 1994). In Loligo bleekeri, mature body size is a trait that shows secondary sexual development, and males generally become larger than females. In our results, mature males showed body size dimorphism in 2005, although such a clear bimodality was not observed in 2004. Because loliginid squid has an annual life span (Boyle & Rodhouse 2005), the bimodality and the difference in size distribution among
years is not due to age classes. The difference in size distribution among years might be
related to oceanographic and biological environment factors such as water temperature and
food availability. Hectocotylus length in 2004 and fin width in 2005 showed a non-linear
relationship with mantle length in Model 1. However, these results were not consistent for
two years and no significant switch point was detected for these body parts in Model 2 and
Model 3 in both years. There was no morphological discontinuity in relation to male body
size in the other four external characters, too (fin length, head width, mantle width, and
tentacle length). In contrast, spermatophore length showed clear discontinuity in relation to
body size in both years. Previous studies have shown that small males tend to adopt
sneaking copulation in the head-to-head position (L. pealei: Hanlon et al. 1997; L. vulgaris
reynaudii: Hanlon et al. 2002). Although the behavioral-switch body size is unknown, small
males with short spermatophore and large males with long spermatophore would adapt for
sneaking copulation in the head-to-head position and for pair copulation in male-parallel
position, respectively. This suggestion is consistent with the results of our observations on
each sperm mass type attached on the different body parts of females.

One possible hypothesis to explain the dimorphism of spermatophore would be
quantitative and morphological adaptation of sperm mass to the morphology of the female’s
sperm-storage organs. Some studies have demonstrated that morphs of male’s genitalia and sperm characters coevolved with the morphology of the seminal receptacle in females (Pitnick et al. 2003, Hosken & Stockley 2004). A further study to examine the morphology and the volume of the respective sperm storage sites would be required to test this hypothesis.

Another possible hypothesis comes from the view of sperm allocation. There is no evidence that spermatophore length and sperm number are related, but short spermatophores in small males may not coincide with the prediction from sperm competition theory assuming “fair raffle principle” that sneaker males should ejaculate relatively more. In loliginid squid, eggs being spawned will meet sperm stored in the oviduct before stored sperm in the seminal receptacle near the mouth. Fertilization success is far lower in head-to-head copulation than in paired male-parallel copulation (Iwata et al. 2005). Such a fertilization mechanism would strictly restrict available eggs for head-to-head copulation. Several studies have shown that males adjust the sperm number released based on the expected number of eggs when available eggs vary among mating (e.g., males release more sperm when mating with larger females; Marconato & Shapiro 1996, Sato et al. 2006). In loliginid squid, the fertilization success of head-to-head
copulation events might not dramatically increase with an increase in sperm number due to a limited availability of eggs, and strategic allocation of sperm to more females than a single female might be adaptive for small males. Furthermore, it has been recently suggested that males under sperm competition invest energy not only in sperm number but also in sperm quality, such as size, velocity and mobility (Leach & Montgoerie 2000, Vladić & Järvi 2001, Uglem et al. 2001). Investment in sperm number must be maintained at some minimum requirement to ensure fertility, but with limited resources available for reproduction, there is likely to be a trade-off between sperm number and quality (Snook 2005). Comparing the sperm number and quality (size, velocity and mortality) in long- and short-type spermatophores would provide more insight into the adaptive value of the spermatophores dimorphism found in this study.

In conclusion, this study reports on dimorphism in ejaculate characteristics in cephalopods for the first time, to our knowledge. The morphological switch in spermatophore length would be associated with the two mating tactics: large males pass sperm to the oviduct opening with long spermatophores by male-parallel copulation, and small males pass sperm to the seminal receptacle with short spermatophores by head-to-head copulation. Our results suggest that alternative reproductive behaviors
promote internal morphological adjustment under the different roles that males play in the
sperm competition game.

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LITERATURE CITED


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reproductive behaviors in the squid *Loligo bleekeri*. Mar Ecol Prog Ser 298:219-228


Figure legends


Fig. 2. *Loligo bleekeri*. The relationship between mantle length and spermatophore length. A: 2004 (n=72). B: 2005 (n = 269). Dashed vertical line shows morphological switch point (A: mantle length 207 mm, B: 221 mm).

Fig. 3. *Loligo bleekeri*. The typical morphology of spermatophores and ejaculated sperm mass. A: Short spermatophore (above) and a drop-like shape ejaculated sperm mass (below, white arrow). Scale bar = 10 mm. B: Long spermatophore (above) and a rope-like shape ejaculated sperm mass (below, white arrow). Scale bar = 10 mm. C: Sperm mass (white arrows) attached around the seminal receptacle (SR) near the beak (BK) of female. Scale bar = 5 mm. D: Sperm mass (white arrow) attached in the oviduct (OV) of a female. The oviduct was dissected to show its inside where sperm mass were attached. Scale bar = 10 mm.
Table 1. *Loligo bleekeri*. Test of significance for $H_0$: $\alpha_2 = 0$ in Model 1 and $H_0$: $\beta_3 = 0$ in Model 2.

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Fig. 1.
Fig. 2.
Fig. 3.