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1 **Testes-size variation within sneaker males of the dusky frillgoby *Bathygobius fuscus***  
2 **(Gobiidae): effects of within-tactic competition**

3

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11

12 Running title: Testes-size variation within sneaker males

13 Sneaking tactic is a typical alternative reproductive tactic that usually results in sperm  
14 competition among males with different tactics. Relatively large testes are a  
15 sneaker-specific trait that has generally been thought to evolve due to sperm competition  
16 between sneaker males and bourgeois males. However, here we present that competition  
17 among sneaker males can also affect testes enlargement in the dusky frillgoby  
18 (*Bathygobius fuscus*) sneaker males. The competitive advantage of focal sneaker males  
19 was experimentally manipulated by placing them in tanks with either relatively smaller or  
20 larger males. Testes enlargement was conspicuous in focal males that were cohoused with  
21 larger males. Smaller sneaker males may invest more in testicular growth because they  
22 are at a competitive disadvantage in the physical contest for sneaking opportunities  
23 among sneaker males and consequently may be confined to making relatively late  
24 intrusions into nests during spawning that have a higher risk of sperm competition.  
25 Another possible reason for the relative size-dependent energy investment in testes may  
26 be increased investment by large sneaker males in aggressive interactions for sneaking  
27 opportunities. This is the first evidence that the testes-size variation among sneaker males  
28 is affected by the competition among sneaker males.

29

30 **KEYWORDS:** alternative reproductive tactics; goby; sneaking; sperm competition; testis

31

## INTRODUCTION

32 Alternative reproductive tactics are an intraspecies variation of reproductive  
33 phenotypes (Gross, 1996), which are maintained because of their contribution to  
34 maximising individual fitness (Koprowski, 1993; Gross, 1996; Shuster & Wade, 2003).  
35 Although tactics are genetically fixed in some species (i.e. alternative strategy: e.g.  
36 Shuster & Wade, 1991; Ryan, Pease & Morris, 1992; Lank *et al.*, 1995), they are  
37 determined by an individual's social situation and environmental condition in most  
38 (Roff, 1996; Tomkins & Hazel, 2007). Differences in reproductive tactics are generally  
39 represented by the differences in reproductive traits, such as body size, colour,  
40 morphology and behaviour, and individuals typically develop tactic-specific traits that  
41 enhance the effectiveness of the reproductive tactics they employ (Brockmann &  
42 Taborsky, 2008). For example, fighting and dispersal tactics are common in many  
43 insect species and fighter males typically have larger bodies or body parts, such as  
44 enlarged mandibles and forelegs, whereas disperser males are smaller but develop long  
45 wings (Brockmann, 2008).

46 Sneaking tactic is a parasitic reproductive behaviour to steal fertilisation  
47 opportunities from males that monopolise females by guarding them (i.e. bourgeois  
48 males; see Taborsky, 1997 for terminology). Sneaker males face a higher risk of sperm  
49 competition compared with bourgeois males because sneaker males always spawn at  
50 the same time or soon after bourgeois males, and thereby generally have relatively large  
51 testes (Taborsky, 1994; Parker, 1998; Simmons, Tomkins & Hunt, 1999; Wada *et al.*,  
52 2005; Zamudio & Chan, 2008) which increase their ejaculate volume (Evans, Pierotti  
53 & Pilastro, 2003; Zbinden *et al.*, 2003; Zbinden, Lurgiader & Bakker, 2004). However,  
54 reproductive competition occurs not only between males with different reproductive  
55 tactics but also among those with the same tactic; for example, there is intense physical

56 competition for sneaking opportunities among sneaker males in some species (Thomaz,  
57 Beall & Burke, 1997; Koseki & Maekawa, 2000; Brockmann, Nguyen & Potts, 2000).  
58 Small males are at a competitive disadvantage in such competition and therefore often  
59 have a low success rate of sneak intrusions. Sneaking behaviour is generally favoured  
60 by inconspicuousness, particularly small body sizes (Gross, 1985); however, selection  
61 may act on body size among sneaker males if the outcome of competitive interactions is  
62 dependent on body size. Such competition within a tactic may lead to differences in  
63 other reproductive and life history traits.

64 The dusky frillgoby *Bathygobius fuscus* is a small marine gobiid fish with male  
65 uniparental care of eggs. Relatively large males (ca. 45–95 mm in total length (TL),  
66 Taru, Kanda & Sunobe, 2002; Takegaki, Kaneko & Matsumoto, 2012) occupy rock  
67 holes or crevices as spawning nests, court females and spawn in pairs in the nests  
68 (nest-holding tactic). Nest-holding males tend eggs until they hatch (ca. 4–5 days).  
69 Conversely, relatively small males (ca. 28–73 mm TL) intrude into spawning nests  
70 while spawning is taking place and attempt to fertilise eggs (sneaking tactic). Sneaker  
71 males change their tactics into nest-holding even during a single breeding season (Taru,  
72 Kanda & Sunobe, 2002), but they do not always do so even when both nests and  
73 females are available (Takegaki, Kaneko & Matsumoto, 2013). Sneaker males have  
74 larger testes relative to their body size than nest-holding males, and even among  
75 sneaker males, smaller males have relatively much larger testes than larger males  
76 (Takegaki, Kaneko & Matsumoto, 2012). Since both large and small sneaker males face  
77 a similar probability of sperm competition against nest-holding males, a possible  
78 reason for the variation in testes size among sneaker males is sperm competition among  
79 sneaker males themselves. In this species, sneaker males frequently compete with other  
80 sneaker males for sneaking opportunities (Takegaki, Kaneko & Matsumoto, 2012).

81 Larger sneaker males have a significant advantage in this competition and thus have a  
82 higher frequency of sneaking attempts and intrusions into nests, though small sneaker  
83 males are able to stay in the nests longer than large sneaker males if they succeeded in  
84 sneak intrusion (Takegaki, Kaneko & Matsumoto, 2012). Female egg-laying in this  
85 species lasts for several hours during which several sneaker males typically intrude into  
86 a nest (Takegaki, Kaneko & Matsumoto, 2012). Because the sperm of this species lives  
87 for several hours after activation in sea water (A. Nakanishi & T. Takegaki,  
88 unpublished data), sperm competition may occur, not only between sneaker males and  
89 nest-holding males but also among sneaker males. Although sneak fertilisation success  
90 is high if its sneaking occurs just after start of spawning (Y. Kanatani & T. Takegaki,  
91 unpublished data), smaller sneaker males are assumed to be forced to make later  
92 incursions into nests than larger males. We hypothesise that the relatively large testes of  
93 small sneaker males are an adaptation to the higher risk of sperm competition these  
94 males experience from both nest-holding males and larger sneaker males. To test this  
95 hypothesis, we examined the effects of competition among sneaker males on their  
96 testes-size variation by cohousing them in tanks with other sneaker males of different  
97 sizes.

98

99

## MATERIALS AND METHODS

100 Experiments were conducted from 19 May to 9 July, 2011 (51 days). Gobies were  
101 collected using hand nets from intertidal pools on the Mie-zaki coast, Nagasaki, Japan  
102 (32° 48' N, 129° 44' E) from 14 to 17 May 2011, which is about 2 weeks before the start  
103 of the spawning season. Fish were sexed on the basis of the shape of their genital  
104 papillae and males and females were kept in separate stock tanks (60 × 30 × 30 cm)  
105 until the start of the experiment.

106 The experiment was conducted in 18 glass tanks ( $45 \times 30 \times 30$  cm) that were all  
107 isolated from each other by opaque screens. Each tank had a 2-cm layer of sand on the  
108 bottom and was supplied with aerated seawater (depth, 15 cm; temperature, 22–28°C).  
109 Each tank was divided into three sections with transparent acrylic boards into which  
110 some holes had been drilled to allow water circulation (Fig. 1). Males ranging from  
111 30.0 to 35.8 mm in TL (mean  $\pm$  SD =  $32.5 \pm 1.7$  mm), the size range of small sneaker  
112 males (Takegaki, Kaneko & Matsumoto, 2012), were used as focal sneaker males and  
113 were cohoused with either larger (SK<sub>L</sub>: 45–50 mm TL) or smaller (SK<sub>S</sub>: 25–28 mm TL)  
114 sneaker males to experimentally manipulate the focal males' competitive advantage  
115 among sneaker males. The two end sections of each tank contained either three focal  
116 sneaker males or a large (67.5–73.5 mm TL) nest-holding male, whereas the middle  
117 section contained three cohoused SK<sub>S</sub> or SK<sub>L</sub> males (Fig. 1). Moreover, the focal  
118 males' third of the tank was divided into three equal sized compartments with opaque  
119 acrylic boards so that focal males could be separated and not see each other. There was  
120 no difference in the body size of the focal males in different compartments at the start of  
121 the experiment (one factor ANOVA,  $F_{2, 51} = 0.21$ ,  $p = 0.81$ ), nor did the locations of  
122 focal males' compartments influence their testes size at the end of the experiment  
123 (Friedman's test: SK<sub>L</sub> treatment,  $p = 0.09$ ; SK<sub>S</sub> treatment,  $p = 0.12$ , both  $n = 27$ ). A  
124 small clay flower pot was placed upside down to serve as a spawning nest in the  
125 nest-holding male's section of each tank. A mature female with a swollen abdomen  
126 (43.6–76.5 mm TL) contained within a transparent acrylic pipe (inside diameter: 45  
127 mm, length: 250 mm) with fine nylon mesh at both ends was introduced to the tank for  
128 9 h every 2 days to stimulate male reproductive behaviour throughout the experiment.  
129 All individuals were fed once a day to satiation, and the leftovers were removed  
130 afterwards. At the end of the experiment, TLs and body weights of all focal males were

131 measured, males were euthanised with an anaesthetic solution (quinaldine, 1250 ppm)  
132 and their testes dissected and weighed (g). Using these measurements, the somatic  
133 weight (body weight - testis weight, g), condition factor [body weight ( $g \times 10^5/TL^3$   
134 (mm)] and growth rate (mm/day) were calculated.

135 Aggressive interactions between focal and cohoused males were recorded using a  
136 digital video camera (HDR-XR500V, Sony) to confirm that competition was occurring  
137 between them and to determine which males had a competitive advantage. Thirty to 35  
138 days after the start of the experiment, we conducted behavioural observations (1 h/fish)  
139 on nine focal males randomly selected from each of the experimental groups. A single  
140 aggressive interaction was defined as a completed set of threat and escape behaviours.

141 All statistical analyses were performed using R (R Development Core Team 2016).  
142 The effect of each treatment (cohousing with SK<sub>S</sub> or SK<sub>L</sub>) on the testes size of focal  
143 males was analysed using generalised linear mixed models with a gamma distribution  
144 with log link function. The explanatory variables were the logarithm of the focal males'  
145 somatic weight and the treatment (SK<sub>S</sub> or SK<sub>L</sub>). Tank and compartment location were  
146 random effects. These analyses were conducted using the lme4 package (Bates *et al.*,  
147 2015) in R. Comparisons of the condition, growth rate and the frequency of attacks of  
148 focal males in the different treatments were performed using the Mann–Whitney U test.

149 This study was performed in accordance with the guideline for ethological  
150 studies by the Japan Ethological Society and the guidelines for the use of fishes in  
151 research by the Ichthyological Society of Japan. No permits were needed from the  
152 Japanese government for experiments involving *B. fuscus*.

153

154

## RESULTS



155 Larger focal males tended to have larger testes (Fig. 2; Table 1). Focal males cohoused  
156 with SK<sub>L</sub> males had larger testes compared with those cohoused with SK<sub>S</sub> males (Fig.  
157 2; Table 1). However, the size of cohoused males (SK<sub>L</sub> or SK<sub>S</sub>) had no effect on focal  
158 male growth rate and body condition (Mann–Whitney U test: growth rate,  $z = -0.15$ ;  
159 condition factor,  $z = -0.11$ ; both  $p > 0.05$ ,  $n = 54$ ; Table 2). Focal males were threatened  
160 by cohoused SK<sub>L</sub> males but not by cohoused SK<sub>S</sub> males (Mann–Whitney U test:  $U =$   
161  $13.5$ ,  $p < 0.01$ ,  $n = 9$ ; Table 2). Conversely, focal males threatened cohoused SK<sub>S</sub> males  
162 but not cohoused SK<sub>L</sub> males ( $U = 4.5$ ,  $p < 0.01$ ,  $n = 9$ ; Table 2).

163

164

## DISCUSSION

165 The results of this study demonstrate that *B. fuscus* sneaker males developed larger  
166 testes when they were at a competitive disadvantage relative to other sneaker males.  
167 This indicates that the enlarged testes of small sneaker males are not caused by their  
168 small body size *per se* but rather because smaller body size reduces their competitive  
169 ability against larger sneaker males. Testes enlargement in sneaker males has generally  
170 been regarded as the outcome of sperm competition between sneaker and bourgeois  
171 males (Parker 1998; Taborsky 2008). However, our results show that it is possible for  
172 the testes size of sneaker males to be affected by competition among sneaker males.

173 One possible reason why the investment in testicular growth made by sneaker  
174 males depends on their competitiveness is the different risk of sperm competition  
175 experienced by small and large sneaker males. Both large and small sneaker males have  
176 relatively larger testes compared to nest-holding males, probably because sneaker  
177 males are always subject to sperm competition from nest-holding males, whereas  
178 nest-holding males are sometimes able to fertilise eggs without successful intrusion by  
179 sneaker males. However, sperm competition is also likely to occur among sneaker

180 males of this species. This is because several sneaker males typically compete to  
181 fertilise eggs during a single spawning event (Takegaki, Kaneko & Matsumoto, 2012)  
182 and because *B. fuscus* sperm remains viable for several hours after activation in sea  
183 water (A. Nakanishi & T. Takegaki, unpublished data). For these reasons, there is a high  
184 possibility that sperm from several sneaker males coexists in nests, even when different  
185 males attempt to sneak fertilisation at different times. Larger sneaker males have a  
186 significant advantage in competition among sneaker males over sneaking  
187 opportunities, whereas the probability of achieving a sneak fertilisation or even  
188 intruding into a nest is extremely low for small males (Takegaki, Kaneko &  
189 Matsumoto, 2012). Furthermore, smaller sneaker males would be expected to  
190 experience a higher risk of sperm competition. This is because the success rate of sneak  
191 fertilisation is high when sneaker males are able to intrude just after start of spawning  
192 (Y. Kanatani & T. Takegaki, unpublished data); however, because of their smaller body  
193 size, small sneaker males are assumed to only be able to intrude into nests relatively late  
194 in the spawning process after fertilisation by other sneaker males has already occurred.  
195 Small sneaker males enlarge testes and thereby probably increase in volume of a single  
196 ejaculate to enhance reproductive success under unfavorable conditions: i.e., low  
197 chance of sneak intrusion and high risk of sperm competition.

198       Another possible explanation for the differential investment in testes growth  
199 between small and large sneaker males is strategic energy allocation by large sneaker  
200 males. Although there are no aggressive interactions among sneaker males in the sand  
201 goby *Pomatoschistus minutus*, this species has variation in testes size among sneaker  
202 males similar to that observed in *B. fuscus* (Kvarnemo, Svensson & Manson, 2010).  
203 Although both large and small sneaker males have the potential to change their tactics  
204 (Takegaki, Svensson & Kvarnemo, 2012), small sneaker males have a little chance of

205 becoming nest-holding males, probably because of their short life span (ca. 1–1.5  
206 years). Therefore, the best way for small sneaker males to enhance their reproductive  
207 success is to invest more in testes growth (Takegaki, Svensson & Kvarnemo, 2012). In  
208 addition, they lack male breeding coloration and are thereby likely to intrude into nests  
209 without being noticed by nest-holder males. In *B. fuscus*, sneaker males do not always  
210 become nest-holders, even when nests and females are available, probably because the  
211 mating success of nest-holding males is so dependent on body size that small sneaker  
212 males are unlikely to obtain high mating success by adopting this tactic (Takegaki,  
213 Kaneko & Matsumoto, 2013). Moreover, in addition to large-size advantage, there is a  
214 small-size advantage in sneaking behaviour in this species: i.e., small sneaker males  
215 can stay in the nests longer than large sneaker males if once they succeeded in sneak  
216 intrusion probably because of low risk of being detected by nest-holder males  
217 (Takegaki, Kaneko & Matsumoto, 2012). Thus, the best way for small male *B. fuscus* to  
218 maximise their reproductive success may be to adopt sneaking tactic and increase  
219 investment in testes growth. Conversely, large sneaker males have a relatively high  
220 possibility of changing tactics even within a single breeding season. Therefore, they  
221 should invest more in body, than testicular, growth so that they can maximise their  
222 mating success after becoming nest-holders. Indeed, even though large sneaker males  
223 have relatively larger testes than nest-holding males, the difference is quite small  
224 (Takegaki, Kaneko & Matsumoto, 2012). However, in this study, there was no  
225 difference in the growth rate of focal males between when they cohoused with large and  
226 small sneaker males. Conversely, large sneaker males had a higher frequency of  
227 aggressive behaviour than small ones. If the differences in testes size among sneaker  
228 males are due to the energy allocation strategy of large sneaker males, it is possible that  
229 their low testes investment is to increase investment not in growth for future tactic

230 change but rather in aggressive competition among sneaker males for potential  
231 sneaking opportunity. Similar trade-offs between investment in testes, as opposed to  
232 traits related to male–male competition, have been reported in the horned beetle  
233 (Simmons & Emlen 2006) and howler monkeys (Dunn et al. 2015).

234 This study demonstrated that the difference in testes investment among *B. fuscus*  
235 sneaker males is affected by the competition among sneaker males. The results strongly  
236 suggest that this could be due to sperm competition among sneaker males, but the  
237 alternate explanation of differential allocation of energy resources cannot be excluded.  
238 In either case, determining the effects of both nest-holder and sneaker males on the  
239 testes enlargement of sneaker males is important to understanding the phenotypic  
240 expression and evolution of testes size of sneaker males. This is because if different  
241 selection pressures act on the same trait, its phenotypic expression would be expected  
242 to be enhanced or suppressed in response to the direction and intensity of each type of  
243 selection pressure.

244

245

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250

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340 Figure legends

341 Fig. 1. Experimental design used to examine the effect of competitor size on the testes  
342 size of *B. fuscus* sneaker males. Focal males were cohoused with (a) larger sneaker  
343 males (SK<sub>L</sub>) or (b) smaller sneaker males (SK<sub>S</sub>), in the presence of a nest-holding male  
344 (NH) with a nest. Dotted lines indicate transparent acrylic screens.

345

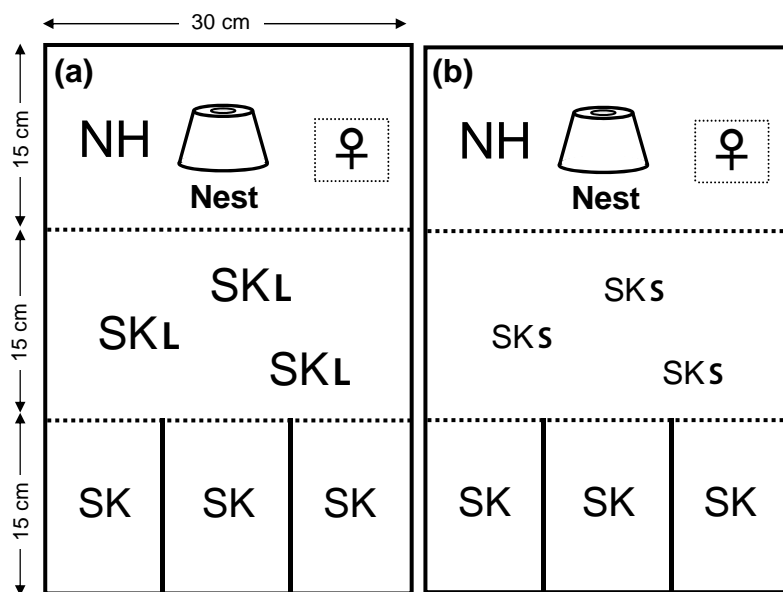
346 Fig. 2. Relationship between somatic weight (log g) and testes weight (log g) of focal *B.*  
347 *fuscus* sneaker males at the end of the experiment. Closed and open circles indicate  
348 focal males cohoused with large (SK<sub>L</sub>, n = 27) or small (SK<sub>S</sub>, n = 27) sneaker males,  
349 respectively.

Table 1. Results of the generalized linear model analysis for the effects of somatic weight and cohoused males on testes weight of the focal males. Estimates  $\pm$  standard errors (SE),  $t$  and  $p$  values are shown.

| Fixed effects                                  | Estimate $\pm$ SE  | $t$   | $p$     |
|--|--------------------|-------|---------|
| Intercept                                      | -4.775 $\pm$ 0.521 | -9.17 | < 0.001 |
| log Somatic weight                             | 1.245 $\pm$ 0.497  | 2.51  | < 0.05  |
| Experimental condition (with SK <sub>S</sub> ) | -0.900 $\pm$ 0.287 | -3.13 | < 0.01  |

Table 2. Comparisons of growth rate, condition factor and frequency of attacks between focal males cohoused with large and small males. Median values and range (in parentheses) are shown.

|                             | Males cohoused with large sneaker (SK <sub>L</sub> ) males | Males cohoused with small sneaker (SK <sub>S</sub> ) males |
|-----------------------------|--|--|
| Growth rate (mm/day)        | 0.05 (0.02–0.10)   | 0.05 (0–0.11)  |
| Condition factor            | 1.02 (0.81–1.19)   | 1.05 (0.77–1.11)   |
| Frequency of attacks (/min) |  |  |
| against cohoused males      | 0 (0)  | 1.0 (0–3)  |
| from cohoused males         | 2.0 (0–5)  | 0 (0)  |



**Figure 1**  
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