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Citation	Marine biology, 166(5), 59 https://doi.org/10.1007/s00227-019-3506-4
Issue Date	2019-05
Doc URL	http://hdl.handle.net/2115/78389
Rights	The final publication is available at link.springer.com
Type	article (author version)
Additional Information	There are other files related to this item in HUSCAP. Check the above URL.
File Information	01_MABI-D-18-00496_R2S.pdf

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Marine Biology

Host selection and ovipositor length in eight sympatric species of sculpins that deposit their eggs into tunicates or sponges
--Manuscript Draft--

Abstract:	Interspecific interactions between parasites and hosts can influence the evolution of behavioural and morphological adaptations of both parasites and their hosts. There is, however, little empirical evidence available regarding the evolution of reproductive traits driven by these interactions. In this paper, we investigated host selection and ovipositor length in nine sympatric marine sculpins that oviposit into tunicates or sponges. Field and genetic studies have revealed host use for eight out of nine species of sculpins investigated here: five species of Pseudoblennius, two species of Furcina and one species of Vellitor. For one species studied (V. minutus), no egg masses could be found. Ovipositor length reflects morphology of host species utilised: six sculpin

species had extremely long ovipositors allowing females to attach eggs to the deep atrium of solitary tunicates, whereas the two species that attached their eggs to the small space of atrial syphon of colonial tunicates and the spongocoel of sponges had short ovipositors. Ovipositor length varied between solitary-tunicate spawners and species with longer ovipositors selected larger tunicates. Since the ancestral form is non-parasitic, the ovipositor evolved as an adaptation to utilise sponges and tunicates as hosts. Sculpins found sympatrically may show host specificity to avoid interspecific competition for spawning niches and ovipositors may have evolved depending on the species and size of host invertebrates.

Original paper

Host selection and ovipositor length in eight sympatric species of sculpins that deposit their eggs into tunicates or sponges

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Short title: Host selection and ovipositor length in sculpins

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1   **Abstract**

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3   behavioural and morphological adaptations of both parasites and their hosts. There is,

4   however, little empirical evidence available regarding the evolution of reproductive traits

5   driven by these interactions. In this paper, we investigated host selection and ovipositor length

6   in nine sympatric marine sculpins that oviposit into tunicates or sponges. Field and genetic

7   studies have revealed host use for eight out of nine species of sculpins investigated here: five

8   species of *Pseudoblennius*, two species of *Furcina* and one species of *Vellitor*. For one species

9   studied (*V. minutus*), no egg masses could be found. Ovipositor length reflects morphology of

10   host species utilised: six sculpin species had extremely long ovipositors allowing females to

11   attach eggs to the deep atrium of solitary tunicates, whereas the two species that attached their

12   eggs to the small space of atrial syphon of colonial tunicates and the spongocoel of sponges

13   had short ovipositors. Ovipositor length varied between solitary-tunicate spawners and species

14   with longer ovipositors selected larger tunicates. Since the ancestral form is non-parasitic, the

15   ovipositor evolved as an adaptation to utilise sponges and tunicates as hosts. Sculpins found

16   sympatrically may show host specificity to avoid interspecific competition for spawning

17   niches and ovipositors may have evolved depending on the species and size of host

18   invertebrates.

19   Keywords: ovipositor; sculpins; tunicates; sponges; marine fish; ostracophils.

Introduction

Sexual selection, arising from interaction between sexes, has considerable influence on

evolutionary adaptations in both sexes, for example, the morphological adaptation of

reproductive traits (Andersson 1994; Birkhead and Møller 1998). However, as yet only a few

studies have examined the evolution of reproductive traits in parasites through the interactions

between parasites and hosts. In parasitic animals that utilise other organisms as oviposition

hosts, female selection and oviposition success can strongly affect the survival of offspring

and thereby parental fitness (e.g. Smith et al. 2001; Mills and Reynolds 2002, 2003; Kitamura

2005; Reichard et al. 2007), whereas hosts often incur costs due to egg deposition (e.g. Peden

and Corbett 1973; Poltev and Mukhametov 2009; Reichard et al. 2006). Therefore, such

interspecifically antagonistic interactions can influence the evolution of behavioural and

morphological adaptations of either or both parasites and their hosts.

Some of the parasitic animals that inhabit aquatic environments preferentially deposit

their eggs in live invertebrates (known as 'ostracophils': Balon 1975; Leung 2014). For

example, bitterlings (Acheilognathinae) deposit their eggs in the gill chambers of freshwater

mussels through their atrial siphon (e.g. Smith et al. 2004; Kitamura 2007), the Japanese

tubesnout (*Aulichthys japonicus*) conceals their eggs in tunicates (Akagawa et al. 2008) and

snailfishes (Liparidae) deposit eggs within the branchial chambers of crabs (e.g. Hunter 1969;

Somerton and Donaldson 1998; Poltev and Mukhametov 2009; Gardner et al. 2016). All of

these species display some degree of host specificity, and various degrees of adaptation of

reproductive traits, for the deposition of their eggs inside invertebrates (reviewed in Leung

2014); for example, the extremely long ovipositors observed in both bitterlings (Smith et al.

2004; Kitamura 2007; Kitamura et al. 2012) and snailfishes (Hunter 1969). Similar

adaptations of reproductive traits through interactions between parasites and hosts have been

44 reported for various parasitic wasps including parasitoids that target free-living insects (Le

45 Ralec et al. 1996), gall-makers (Ghara et al. 2011) and figs (Elias et al. 2018). These

46 ostracophilous fishes and parasitic wasps have shown that sexual traits can be inter- and

47 intraspecifically variable, due to interactions between parasites and hosts. However, empirical

48 evidence regarding these variations in reproductive traits and host selection remain scarce.

49   Sculpins (Teleostei: Cottidae), which belong to the Cottoidea superfamily, are a large

50 group of fish that have 70 genera and 282 species worldwide, and primarily inhabit boreal and

51 cold temperate regions (Nelson et al. 2016). Although evidence is scarce, several species of

52 Cottoidea are known to deposit their eggs inside alive invertebrates (reviewed in Abe and

53 Munehara 2009; Munehara et al. 2011). For example, the little dragon sculpin *Blepsias*

54 *cirrhosus* and the bigmouth sculpin *Hemitripterus bolini* utilise sponges as their spawning

55 host (Munehara 1991; Busby et al. 2012). It has also been reported that four sculpin species in

56 the genus *Pseudoblennius* that inhabit temperate waters seem to be ostracophils (Uchida 1932,

57 1979; Shinomiya and Ikemoto 1987; Okamura and Amaoka 1997; Nishida et al. 2008; Uryu

58 2011). According to these descriptive studies and to underwater photographs taken by divers,

59 these species are all copulating species in which females deposit their eggs exclusively inside

60 tunicates or sponges using their ovipositors (Shiogaki and Dotsu 1974; Shinomiya 1985; Abe

61 and Munehara 2009; Nishida et al. 2008; Uryu 2011). In addition, during a preliminary study

62 of marine sculpins in the nearshore reef of Sado Island, located in the Sea of Japan, it was

63 found that there may be nine species of potentially ostracophilous sculpins (five species of

64 *Pseudoblennius*, two species of *Furcina* and two species of *Vellitor*). They all develop

65 ovipositors and occur sympatrically in shallow water of the island (Awata 2015, 2017).

66 Therefore, these sculpins may be ideal for an examination of the evolution of interspecific

67 variation in reproductive traits that are driven by interactions between parasites and hosts.

68 However, no studies have demonstrated host selection and ovipositor morphology in sculpins,

69   with the exception of one study that showed host tunicate selection by *Pseudoblennius*

70   *percoides* (Nishida et al. 2008).

71   In this study, we investigated interspecific variation in host selection and ovipositor

72   length in nine sympatric species of marine sculpins that deposit their eggs into tunicates or

73   sponges on the coast of Sado Island, northern Japan. We hypothesise that species-specific host

74   selection by sculpin females drives the evolution of ovipositor morphology. To confirm our

75   hypothesis, we tested three predictions. Firstly, that the nine ostracophilous sculpin species

76   would utilise different species and sizes of host tunicates or sponges to reduce interspecific

77   competition for hosts (Reichard et al. 2007). This is because these sculpins co-occurred at the

78   study sites. Secondly, that only ostracophilous sculpins, and not species that lay eggs on rock

79   surfaces, would have elongated ovipositors. The morphological and molecular phylogeny of

80   the sculpins suggests that ostracophilous sculpins have evolved from non-ostracophilous

81   sculpins (Yabe 1985; Abe and Munehara 2009; Koya et al. 2011; Knope 2013). Therefore, the

82   development of the ovipositor is likely related to such unique spawning behaviour. Finally,

83   that ovipositor length would reflect the species and size of the hosts utilised.

84   **Materials and methods**

85   **Study sites**

86   Fieldwork using self-contained underwater breathing apparatus (SCUBA) was conducted on

87   the coast of Sado Island in the Sea of Japan. The island is located about 45 km off the

88   Japanese mainland coastal city of Niigata (Fig. 1a). The amplitude of the tides around the

89   island is relatively low, ranging from only 0.2 to 0.3 m. Four sites (Fig. 1b: Kitakoura: 38° 16′

90   26.90″ N, 138° 30′ 34.42″ E; Mushizaki: 38° 14′ 58.66″ N, 138° 30′ 24.37″ E; Tassya: 38° 04'

91   28.64″ N, 138° 14′ 40.81″ E and; Kotoura: 37° 48′ 13.43″ N, 138° 15′ 08.26″ E) were selected

92 for fieldwork. All of the host invertebrates and ostracophilous sculpin species were abundant

93 at the study sites, with the exception of *V. minutus* that was only found at Tassya (Awata

94 2017). The four study sites were located on rocky shores where seaweed grows abundantly.

95 **Fish study species**

96 There were nine species of potentially ostracophilous sculpins in the nearshore reef of Sado

97 Island (Nakabo and Kai 2013; Awata 2015, 2017). Microhabitat preferences differ between

98 species (Iwata 1983; Awata 2017), but all species occurred sympatrically in rocky shallow

99 water ranging in depth from 1 to 20 m. They are copulating species and their fertilisation

100 mode is internal gametic association (IGA: Munehara et al. 1989), where fertilisation occurs

101 only when eggs are released into the seawater (Shiogaki and Dotsu 1974; Kimura et al. 1987,

102 1988; Koya et al. 2015). Eggs that are released into seawater are spherical of 1.3–2.0 mm in

103 diameter, demersal and adhesive, forming egg masses of up to 500 eggs (e.g. Kimura et al.

104 1987, 1988; Nishida et al. 2008). There are nine species in three genera, *Furcina*,

105 *Pseudoblennius* and *Vellitor*, which form a closely related and monophyletic group (Yabe

106 1985; Munehara et al. 2011). The reproductive ecology is similar among species, but their

107 body size and shape are notably different: *Pseudoblennius* sp. ("Kirin-anahaze" in Japanese,

108 see Nakabo and Kai 2013) and *Ps. percoides* are large sized (130–170 mm in standard length,

109 SL), *Ps. cottoides*, *Ps. marmoratus* and *Ps. zonostigma* are medium sized (80–100 mm SL)

110 and *F. ishikawae, F. osimae, V. centropomus* and *V. minutus* are small sized sculpins (40–70

111 mm SL; Okamura and Amaoka 1997; Munehara et al. 2011; Nakabo and Kai 2013; Awata

112 2017).

113 **Fish collection and measurement of ovipositor length**

114 To examine the ovipositor length of the nine species of ostracophilous sculpins, adult females

6

115 were captured using hand-nets while SCUBA diving during the spawning season, from late

116 November to late April in 2014, 2015 and 2016, in depth of 2 to 15 m at all four study sites

117 (Fig. 1b). The fish were transported in aerated coolers to the laboratory at the Sado Marine

118 Biological Station (SMBS), Niigata University, Sado, Japan. Species identification of sculpins

119 was based on that of Nakabo and Kai (2013). Fish were anaesthetised with a solution of

120 MS222 or 2-phenoxyethanol buffered with seawater, and their SL (to 1 mm) was measured

121 using a scale. The ovipositor was induced to its maximum extrusion by gently pressing the

122 belly of the fish between ventral fins and anus. The ovipositor was photographed alongside a

123 scale, and ovipositor length (OL) was measured to the nearest 1 mm from the apex to the

124 ventral edge of the ovipositor using image analysis software ImageJ 1.50i. A part of the right

125 pectoral fin was clipped from each individual and preserved in 99 % ethanol for genetic

126 analysis. Of the 124 females analysed, 40 were released after the operation and 84 were used

127 for other studies after sacrification under deep anaesthesia (taxonomy and sperm analyses:

128 Awata 2015, 2017; histology: Koya et al. 2015).

129   To determine whether only ostracophilous sculpins have evolved elongated

130 ovipositors, we compared the OL of ostracophilous sculpins and sculpin species that oviposit

131 on the rock surface. We captured 13 females of four non-ostracophilous sculpin species

132 (*Alcichthys alcicornis*, *Bero elegans*, *Icelinus pietschi* and *Radulinopsis derjavini*) using

133 SCUBA at Usujiri (41° 56′ 11.85″ N, 140° 56′ 54.66″ E; Fig. 1a), southern Hokkaido, Japan

134 and at Kotoura, Sado Island, Japan, and measured their SL and OL.

135 **Collection of egg masses deposited in host invertebrates**

136 Egg masses deposited in the spongocoels of sponges and in the common atrial syphons of

137 colonial tunicates were collected in April and May 2012, from March to May 2013 and from

138 February to April 2014 at Kotoura and Kitakoura (Fig. 1b). Egg masses were externally

7

139   visible, and therefore only whole colonies of sponges and colonial tunicates that contained

140   egg masses were collected using scrapers. The colonies that contained eggs were distributed

141   across rock wall habitats at depths of 1–12 m. The sea water temperature was 9–14 °C.

142   Because the egg masses deposited in the atria of solitary tunicates were rarely

143   visible from outside, we haphazardly collected solitary tunicates ($n = 1212$) in December

144   2011, December 2012 and from December 2013 to May 2014 (approximately once a month)

145   at Kitakoura, Mushizaki and Tassya (Fig. 1b). Of these, 1129 (93 %) tunicates were captured

146   from the wall of Kitakoura Port at a depth of 0.5–4.0 m (Fig. 1c), where the wall was covered

147   with in excess of a hundred thousand solitary tunicates. The remaining samples were collected

148   at 8–18 m depth of off Kitakoura Port, at 8–30 m depth of Mushizaki and at 2–5 m of Tassya.

149   The sea water temperature ranged from 9 to 16 °C (the coldest being in February).

150   All tunicates, sponges and egg masses were transferred to the laboratory in aerated

151   coolers. Sponges and colonial tunicates were cut from the osculum and common atrial siphon,

152   respectively, to the spongocoel and common atrium. The length between the open top and the

153   top of the egg mass was measured using a scale (defined as "egg mass position"). Solitary

154   tunicates were photographed with a scale and their long diameter (LD) and short diameter

155   (SD) were later measured using ImageJ 1.50i software. After being photographed, tunicates

156   were carefully cut in half vertically using a kitchen knife, in order to avoid damaging the egg

157   masses. When egg masses were found inside tunicates, opened tunicates were photographed

158   again with a scale to measure the length from entrance of the atrial siphon to the nearest edge

159   of the egg masses (also defined as "egg mass position"). It is natural to expect that utilisation

160   of larger tunicates will require longer ovipositors, and thus egg mass position will be deeper

161   when larger tunicates are used as hosts. The LD was used as an indicator of the body size of

162   tunicates, given that their LD and SD are strongly correlated (Pearson's $r = 0.92$, $p < 0.0001$,

163    $n = 1212$).

164   Each egg mass removed from the host was counted and transferred to a box-shaped

165   net cage (12.5 × 13.0 × 8.0 cm, mesh size: 0.5 mm, s-5330, Sudo & Company, Inc., Nagoya,

166   Japan) that was fixed to the surface of a 90 × 45 × 45 cm tank at the SMBS. Each egg mass

167   was separately incubated for 1–20 days in the cage at 15 °C. Hatched larvae or late stage eggs

168   (just before hatching) were preserved in 99 % ethanol after being killed by an overdose of

169   anaesthesia.

170   Note that egg masses of the Japanese tubesnout *Aulichthys japonicas* were also

171   collected during this study, but the details of this will be published in a later paper.

172   **Genetic identification of parent species of egg masses**

173   To infer the parental species of the egg masses, 1–3 larvae per egg mass and fin-clip samples

174   of nine potentially ostracophilous sculpins, with the addition of *Jordania zonope* as an

175   outgroup, were chosen for use in genetic analyses. Fin-clip samples were obtained from 2011–

176   2017 (Table S1, Supplementary Information). Whole genomic DNA was extracted from 148

177   adults and from 223 larvae (including late stage eggs) of 120 egg masses, using a Gentra

178   Puregene Tissue Kit (Qiagen, Hilden, Germany). Samples were PCR amplified for the

179   mitochondrial cytochrome *b* gene (*cytb*): GLUDG-L: 5'-YGACTTGAARAACCAYCGTTG-

180   3' and CB3-H: 5'-GGCAAATAGGAARTATCATTC-3' (Palumbi 1996; Knope 2013). PCR

181   amplifications were performed at a 15 µL volume using 7.5 µL of 2 × Go Taq Green Master

182   Mix (Promega, WI, USA), 0.6 µL (5 µM) of each primer, 1.5 µL ca. 50 ng/µL of template

183   DNA and 4.8 µL of nuclease free water. PCR thermal cycling was performed using the

184   following protocol for *cytb*: 2 min of initial denaturation at 95 °C, followed by 35 cycles of

185   94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 min. PCR amplifications were successful

186   except for some *Ps. cottoides* and *Ps. zonostigma* and many of the egg masses, for which

187   sequences corresponding to the primers were permutated. Therefore, these samples were PCR

9

188   amplified using another primer set for *cytb*: H15915: 5'-CAACGATCTCCGGTTT-3' and

189   L14724: 5'-GTGACTTGAAAAACCA-3' (Schmidt and Gold 1993). The 800 bp sequences

190   amplified by this primer set overlapped with those amplified by the former primer set. PCR

191   products were electrophoresed on 1.5 % agarose gel stained with GelRed™ (Biotium, Inc.,

192   CA, USA). Positive amplicons (evidenced by a clear single band of the correct size) were

193   enzymatically cleaned with illustra™ ExoStar™ (GE Healthcare UK Limited, Little Chalfont,

194   Buckinghamshire, UK). Double stranded PCR products were sequenced directly with the

195   same primers used for the PCR amplifications at Macrogen Japan Corp. (Kyoto, Japan). All

196   sequences were aligned using the ClustalW algorithm implemented in MEGA ver. 7.0.14.

197   (Kumar et al. 2016).

198   The phylogenetic relationships of nine potential parental species ($n = 146$) and

199   *Jordania zonope* ($n = 2$, as the root for the tree) were reconstructed using 800 bp *cytb*

200   sequences with MEGA ver. 7.0.14, and the Maximum Likelihood method was employed

201   using the Tamura Nei model settings (Tamura and Nei 1993) and 1000 bootstrap replicates.

202   After assessing the usefulness of the sequences for species identification (Fig. S1,

203   Supplementary Information; all these sequences available on GenBank, Table S1), the

204   parental species of egg masses were estimated using the reconstructed phylogeny of the

205   parental species and egg masses. Of 223 eggs analysed, 205 were identified using 800 bp *cytb*

206   sequences, and 18 were identified using 628 bp, due to the low quality of the sequencing data.

207   **Data analyses**

208   Statistical analyses were performed using SPSS ver. 23.0 (IBM Corp., Armonk, NY, USA).

209   Data were analysed using parametric statistics throughout. All reported *p*-values were two

210   tailed, and results were considered statistically significant at *p*-values of < 0.05. As we did not

211   obtain all data from all sponges and tunicates, sample size varied between analyses. To

212   determine whether the relative OL differed between ostracophilous and non-ostracophilous

213   sculpins, we used an analysis of covariance (ANCOVA) with log-transformed OL as the

214   dependent variable and deposition site (sponge, colonial tunicate, solitary tunicate and rock

215   surface) as the independent variable and log-transformed SL as the covariate, including their

216   interaction (full model). If the interaction term (deposition site $\times$ log SL) was not statistically

217   significant, then a reduced model was constructed by reducing the interaction term.

218   **Results**

219   **Egg masses deposited in sponges and colonial tunicates**

220   Nine egg masses were obtained from sponges (Demospongiae) of nine colonies. Since each

221   colony was small and formed mostly only one spongocoel, multiple egg masses were not

222   found in sponges sampled (Fig. 2a). In contrast, the colony size and shape of colonial

223   tunicates (Didemnidae) were variable, ranging from 20 to 100 mm in maximum width, each

224   of which had 1–38 exhalant syphons and contained 1–5 sculpin egg masses at similar or

225   different developmental stages (Fig. 2b, c). In total, 34 egg masses were obtained from the

226   colonial tunicates of 22 colonies.

227   **Egg masses deposited in solitary tunicates**

228   Of 1212 solitary tunicates collected, seven species were identified. The most commonly

229   occurring was *Halocynthia ritteri* (55.9 %, $n = 678$), followed by *Polycarpa cryptocarpa*

230   *kroboja* (25.3 %, $n = 307$), *Cnemidocarpa irene* (11.6 %, $n = 141$), *Pyura sacciformis* (3.5 %,

231    $n = 42$), *Microcosmus hartmeyeri* (2.0 %, $n = 24$), *H. roretzi* (1.6 %, $n = 19$) and *Herdmania*

232   *japonica* (0.1 %, $n = 1$). Although a considerable number of tunicates were collected, sculpins

233   only utilised three species (Fig. 2d–h). The two largest and the smallest species of tunicates

11

234 were not used by the sculpins (Fig. 3). The second most abundant tunicate *Po. c. kroboja* was

235 never used by sculpins, although notably overlapped in size with the most abundant and most

236 frequently used tunicate *H. ritteri* (Fig. 3).

237 Of the 89 sculpin egg masses collected, > 90 % were found in *H. ritteri* and the

238 remainder were found in *Py. sacciformis* and *H. roretzi* (Fig. 2f–h; Fig. 3). Although the

239 abundance of *H. ritteri* was more than 16 times greater than that of *Py. sacciformis* at the

240 study sites, the proportion of parasitized tunicates was equal ($n_{with\ eggs}/n_{without\ eggs}$ = 75/593 for

241 *H. ritteri*; 6/30 for *Py. sacciformis*: Fisher's exact test, p = 0.29). Note that sculpin eggs were

242 only found in the atria of tunicates, indicating that the ovipositors were inserted through the

243 atrial syphon of the tunicates.

244 **Parental species of egg mass**

245 The reconstructed phylogenetic relationships of the potentially parental species and 120 egg

246 masses showed that sponges were utilised by *Ps. marmoratus* (n = 4 egg masses, Fig. 2a) and

247 *V. centropomus* (n = 4) as spawning hosts (details in Table S2). All 26 egg masses deposited in

248 colonial tunicates were assigned to *V. centropomus* (Fig. 2b). Solitary tunicates were used by

249 six sculpin species (Table 1; Fig. 2d–h; details in Table S3): egg masses of *Ps. cottoides* were

250 the most frequent (n = 52 egg masses), followed by those of *Ps. zonostigma* (n = 13),

251 *Pseudoblennius* sp. "Kirin-anahaze" (n = 9), *Ps. percoides* (n = 8), *F. osimae* (n = 3) and *F.*

252 *ishikawae* (n =1). Five of six species mainly employed *H. ritteri* as their hosts (Table 1; Fig.

253 2f). *Pseudoblennius cottoides* and *Ps. percoides* also utilised *Py. sacciformis*. Egg masses

254 deposited in *H. roretzi* were identified as belonging to *Pseudoblennius* sp. "Kirin-anahaze"

255 (Fig. 2g). *Furcina osimae* only utilised *Py. sacciformis* as the tunicate host (Fig. 2h). No egg

256 masses were genetically assigned to *V. minutus* and so further analyses excluded this species.

12

Egg mass position in different hosts

Since egg masses were visible from the outside of sponges and colonial tunicates, but were

not for solitary tunicates, the egg mass position would be expected to differ between the hosts.

As expected, the egg masses were deposited significantly deeper in solitary tunicates ($16.44 \pm$

6.39 mm, $n = 73$) than in sponges (2.70 ± 1.69 mm, $n = 6$) and colonial tunicates (2.02 ± 1.79

mm, $n = 11$) (one-way ANOVA, $F_{2,87} = 40.46$, $p < 0.0001$; Tukey's HSD, $p < 0.0001$ for both).

The egg mass position did not differ between sponges and colonial tunicates (Tukey's HSD, p

$= 0.97$). This indicates that ovipositor length likely differs between sculpin species using

solitary tunicates and those using sponges and colonial tunicates.

Comparison of ovipositor length between fish using different deposition sites

An ANCOVA analysis showed that the interaction between deposition site and log SL did not

have a significant effect on log OL (full model, $F_{3,129} = 2.09$, $p = 0.11$), and overall, larger

species/individuals had longer ovipositors (reduced model, $F_{1,132} = 176.77$, $p < 0.0001$; Fig.

4a). As predicted, the relative OL was significantly related to deposition site (reduced model,

$F_{3,132} = 557.23$, $p < 0.0001$; Fig. 4a). The relative OL of the ostracophilous sculpins was

significantly longer than that of the non-ostracophilous sculpins (Fig. 4b–e). Moreover, the

sculpin species that use solitary tunicates as hosts had much longer OLs (ca. 20 % of SL) than

that of those using colonial tunicates and sponges. Although the relative OL differed

significantly between *V. centropomus* (ca. 5 % of SL) and *Ps. marmoratus* (ca. 3 % of SL), the

absolute OL did not differ between them (*t*-test, $t = 0.99$, $p = 0.33$; Fig. 4a–c).

Ovipositor length and the size of solitary tunicates used by sculpins

The body size of the six parental species that utilised solitary tunicates as hosts differed

significantly among species (one-way ANOVA, $F_{5,89} = 166.84$, $p < 0.0001$; Fig. 5a). Female

13

280   *Pseudoblennius* sp. "Kirin-anahaze" were the largest, and two species in the *Furcina* genus

281   were the smallest of all six species. Overall, larger species had longer ovipositors ($F_{5,89} =$

282   28.05, $p < 0.0001$; Fig. 5b), but the OL of *Ps. percoides* was significantly shorter than that of

283   *Ps. cottoides* despite its larger body size (also see Fig. 4a).

284   As expected, egg mass position was deeper when larger tunicates were used as hosts

285   (Pearson's $r = 0.49$, $p < 0.0001$, $n = 72$). The LD of tunicates used as hosts and the egg mass

286   position were significantly different among sculpin species (LD: Fig. 5c; one-way ANOVA,

287   $F_{4,79} = 8.81$ $p < 0.0001$; egg mass position: Fig. 5d; $F_{4,68} = 7.05$, $p < 0.0001$). Consistent with

288   the expectation that utilisation of larger tunicates will require longer ovipositors, the LD of

289   tunicates used as hosts and the egg mass position were closely related to the sculpin OL (Fig

290   5); *Pseudoblennius* sp. "Kirin-anahaze", *Ps. cottoides* and *Ps. zonostigma* that have longer

291   ovipositors spawned in a deeper position in larger tunicates, and the genus *Furcina* with a

292   smaller body size and a shorter ovipositor consequently utilised smaller tunicates.

293   *Pseudoblennius percoides*, which has a larger body size but a shorter ovipositor, selected

294   smaller tunicates as spawning hosts.

295   *Pseudoblennius* sp. "Kirin-anahaze", *Ps. cottoides* and *Ps. zonostigma* used similar-

296   sized *H. ritteri* tunicates as hosts (Fig. 5c), indicating severe competitions for hosts among the

297   three species. However, if host *H. ritteri* is abundant and sculpins use the most common size

298   of tunicates, competition for hosts would be weakened. Indeed, *H. ritteri* was used by only

299   11 % of all individuals (Fig. 3d). Additionally, the average and variance of host size were

300   similar between unused *H. ritteri* (84.20 LD ± 20.31, $n = 595$) and that used by

301   *Pseudoblennius* sp. "Kirin-anahaze" (92.84 LD ± 10.37, $n = 7$, t-test, $t = 1.12$, $p = 0.26$; F test

302   to compare two variances, $F = 3.84$, $p = 0.09$), *Ps. cottoides* (87.16 LD ± 18.38, $n = 46$, $t =$

303   0.96, $p = 0.34$; $F = 1.22$, $p = 0.41$) or *Ps. zonostigma* (although slightly larger tunicates were

304   used: 99.12 LD ± 17.37, $n = 11$, $t = 2.42$, $p = 0.02$; $F = 1.37$, $p = 0.61$). These show that these

305   three sculpins used the most common size of *H. ritteri* (Fig. 3d).

306   **Discussion**

307   **Host specificity**

308   In this study, we identified oviposition hosts in eight out of the nine ostracophilous sculpins

309   examined. While a number of researchers and divers have observed spawning behaviours of

310   some of these sculpins, such as *Ps. marmoratus*, *Ps. percoides* and *Ps. zonostigma*

311   (Shinomiya 1985; Okamura and Amaoka 1997; Abe and Munehara 2009; Uryu 2011), so far

312   only one study has reported host tunicate selection by *Ps. percoides* (Nishida et al. 2008).

313   Thus, to our knowledge, this is the first study to confirm the host species used by these eight

314   sculpin species, including *Ps. percoides*. There are three patterns of host use by sculpins, such

315   as species that mainly use colonial tunicates, those that only use sponges and those that only

316   use solitary tunicates for oviposition. This result mostly confirms our first prediction, that

317   ostracophilous sculpins would show host specificity. However, as found in ostracophilous

318   bitterlings (Reichard et al. 2007), sculpins also showed variation in their level of specificity.

319   *Pseudoblennius marmoratus* oviposited only in the spongocoel of sponges, but *V.*

320   *centropomus* utilised colonial tunicates and sponges for oviposition. Eggs of six sculpin

321   species were found in solitary tunicates, but three of these fishes used only a single tunicate

322   species and the others used two tunicate species.

323   Egg mass numbers for each sculpin species collected in this study mostly

324   corresponded to the abundance of parental species at the study sites (see Fig. 4, Table 1).

325   Although we captured most tunicates on the wall of Kitakoura Port, the consistency of

326   appearance frequencies between eggs and parental species implies that the six sculpin species

327   do not avoid the artificial habitat for spawning. Since eggs of *V. minutus* were not found and

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328   only one egg mass of *F. ishikawae* was collected, further field sampling including different

329   sites and seasons are needed to clarify their host use.

330   Sponges are only distantly related to colonial tunicates (e.g. Dunn et al. 2014). Their

331   body structures are therefore notably different. However, they share morphological

332   similarities: both have myriad brachial apertures and shared, common cloacal apertures. These

333   similarities, especially the common structure of the cloacal apertures, may allow female *V.*

334   *centropomus* to utilise both colonial tunicates and sponges as hosts. To our knowledge, it is

335   unlikely that any other fishes utilise such distantly related taxa. *Pseudoblennius marmoratus*

336   only oviposit in sponges, which is consistent with previous underwater observations (Uryu

337   2011). It remains unclear whether *Ps. marmoratus* also utilises colonial tunicates. To confirm

338   this, more information on its spawning behaviour in the field needs to be gathered. These two

339   sculpin species have short and similarly sized ovipositors, suggesting that they are specialised

340   in ovipositing in colonial tunicates and sponges.

341   We found that sculpins used only three out of seven species of solitary tunicates as

342   hosts, even though many species of tunicates co-occurred at the study sites. There are three

343   possibilities to explain sculpin host selection: 1) the size of the tunicates, 2) the internal

344   structure of the tunicates and 3) chemical substances in the atria of the tunicates. First,

345   sculpins may select for tunicates of optimal size. Solitary tunicate spawners have developed

346   long ovipositors, which may allow the placement of their eggs in the atrium of tunicates via

347   the atrial siphon. The elongation of the ovipositor is limited by the cost associated with

348   growing and maintaining a long ovipositor. Sculpin females did not use the large tunicates *M.*

349   *hartmeyeri* and *Herdmania japonica*, probably due to the excessively long distance from the

350   entrance of atrial siphon to the atrium of the tunicate. In contrast, the small atrium and narrow

351   entrance of the atrial siphon of the smallest tunicate, *C. irene*, was too small to allow sculpins

352   to use them as hosts.

353   Second, the internal structure of tunicates may be related to host selection by

354   sculpins. Our data showed that the size range of unused tunicate *Po. c. kroboja* greatly

355   overlapped with that of the commonly used tunicate *H. ritteri*. The unused *Po. c. kroboja*

356   tunicates are more compressed compared with the *H. ritteri* tunicates (Awata S. pers. obs.),

357   suggesting that sculpins do not utilise *Po. c. kroboja* due to the small inner space of their atria.

358   Finally, substances in the atria of tunicates might also affect host use by female sculpins. A

359   number of tunicates produce chemical defences, such as high sulfuric acid and high vanadium

360   content, which may reduce their predation risks (e.g. Stoecker 1980; Paul and Puglisi 2004;

361   Paul et al. 2006). *Polycarpa cryptocarpa kroboja* has a uniquely strong smell (Awata S. pers.

362   obs.) compared to the other tunicate species dissected. Although the chemical components

363   responsible for this smell were not identified, they may have adverse effects on the fish eggs.

364   Our aquarium experiments have demonstrated that when *Ps. cottoides* females with ripe eggs

365   were allowed to choose *H. ritteri* and *Po. c. kroboja* of the similar size, they spent more time

366   inspecting *H. ritteri*, and eggs were deposited only in *H. ritteri* (Awata S, Igarashi N, unpub.

367   data). This indicates that the female sculpins likely discriminate between *H. ritteri* and *Po. c.*

368   *kroboja* prior to spawning, and clearly prefer the former over the latter as oviposition hosts. It

369   remains to be experimentally determined which combinations of these proximate factors such

370   as tunicate size, structure and chemical substances affect host selection by female sculpins.

371   **Ovipositor length**

372   In accordance with our second prediction, only ostracophilous sculpins had elongated

373   ovipositors, while non-ostracophilous sculpins lacked this feature. Since non-ostracophilous

374   sculpins have been suggested to be the ancestral state (Yabe 1985; Koya et al. 2011;

375   Munehara et al. 2011; Knope 2013), we can conclude that the elongated ovipositor has

376   evolved as an adaptation to the ostracophilous reproduction strategy. The ovipositor in species

377   using solitary tunicates as hosts was much longer than that in species using colonial tunicates

378   and sponges. The position of eggs oviposited in colonial tunicates and sponges were as short

379   as they were observed to be from the outside, and perfectly matched the short ovipositor

380   length of *V. centropomus* and *Ps. marmoratus*. In contrast, sculpins that oviposited in solitary

381   tunicates had extremely long ovipositors, which in turn matched the distance from the

382   entrance of the atrial siphon to the edge of the egg masses. These results are consistent with

383   the third prediction, that ovipositor length would reflect the species and size of hosts utilised.

384   Both the branchial and atrial siphons of solitary tunicates close immediately by

385   ejecting the water from their body when they detect external stimuli such as vibrations in the

386   water. In contrast, sponges and colonial tunicates shrink a little after the detection of such

387   stimuli (Awata S. pers. obs.). In response to such host behaviour, female sculpins that use

388   solitary tunicates as hosts insert their ovipositors rapidly into the atrium (Movie S1, S2,

389   Supplementary Information). In contrast, species that use sponges as hosts oviposit slowly

390   (Movie S3). Based on these observations, we suggest that spawning behaviour and ovipositor

391   length may have adapted to suit the different hosts.

392   **Competition among sculpin species over hosts**

393   In this study, six species of sculpins were determined to use solitary tunicates as hosts.

394   *Furcina osimae* only used the tunicate *Py. sacciformis* as a spawning host, but the other five

395   species mainly deposited their eggs into *H. ritteri*. This suggests these five species did not

396   prefer specific host tunicates. However, their host use may be also related to the size and

397   species of the host tunicates. Indeed, two smallest species of genus *Furcina*, which have

398   shorter ovipositors, used smaller tunicates; the largest *Pseudoblennius* sp. "Kirin-anahaze",

399   which has longer ovipositors, spawned in larger tunicates. In contrast, the ovipositor of *Ps.*

400   *percoides* was shorter than that of *Ps. cottoides*, despite its larger body, and matches the size

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401   of the host tunicates. Medium-sized sculpins such as *Ps. cottoides* and *Ps. zonostigma* had

402   medium-sized ovipositors but used larger tunicates as hosts. Thus, as expected from our first

403   prediction, sculpins likely choose different sized hosts that fit their ovipositor size, probably

404   to reduce interspecific competition for hosts (Reichard et al. 2007).

405   However, three sculpins (*Pseudoblennius* sp. "Kirin-anahaze", *Ps. cottoides* and *Ps.*

406   *zonostigma*) showed similar host choice in terms of species and size, indicating that

407   interspecific competition for hosts may be unavoidable (Kitamura 2007; Reichard et al. 2007).

408   Previous studies have shown that host utilisation by female bitterlings may be determined by

409   host availability, as well as by host preference and interspecific competition among bitterlings

410   (Kitamura 2007; Reichard et al. 2007). In this study, the most abundant host, *H. ritteri*, was

411   used by only 11 % of all individuals. Furthermore, these three sculpins used the most common

412   size of tunicates (65–105 mm in LD of host *H. ritteri*; see Fig. 3d), although *Ps. zonostigma*

413   oviposited into somewhat larger tunicates. Therefore, their host preferences are strongly

414   related to host availability, and the ovipositor length of those three species may have been

415   shaped by the size of the most abundantly available tunicates. Further experimental studies

416   are needed to identify the "real" host size and species preference by controlling for host

417   availability and interspecific competition among ostracophilous sculpins (Reichard et al.

418   2007). Since most *Pseudoblennius* species are piscivores (Okamura and Amaoka 1997; Uryu

419   2011), it would be necessary to take the risk of predation of smaller species and individuals by

420   larger ones into account when determining interspecific competition at the spawning sites.

421   **Costs and benefits for ostracophilous sculpins**

422   There are at least three potential benefits for female sculpins that use tunicates and sponges as

423   oviposition hosts. First, the hosts may provide suitable environments for embryonic

424   development (Hunter 1969; Munehara 1991; Spence and Smith 2013). Both tunicates and

425   sponges are filter feeders that draw water and food particles through incurrent siphon or

426   canals and excrete the filtered water through atria and excurrent siphons, or through

427   spongocoels and oscula. Parental care by fishes that exclusively exhibit care for their eggs

428   mainly consists of fanning and mouthing, which are assumed to increase the oxygen supply

429   and to help to remove dead eggs and dust particles (e.g. Keenleyside 1991). Since filtered

430   water constantly flows through the atria of tunicates and the spongocoels of sponges where

431   the eggs are deposited, the hosts may inadvertently serve a similar function to fanning and

432   mouthing for the eggs. Furthermore, sponges may provide another benefit to egg development

433   in sculpins. Many studies have reported that sponges contain compounds with antibacterial

434   and antifungal properties (e.g. reviewed in Mehbub et al. 2014). Considering the absence of

435   dead eggs in sponges, the antibacterial and antifungal activities of sponges may increase the

436   survival rate of sculpin eggs. Although dead, un-decayed eggs of the little dragon sculpin *B.*

437   *cirrhosus* have been found to be present in sponge tissues (Munehara 1991), such eggs were

438   not found in our study, perhaps due to the difference in embryonic periods (< 1 month in our

439   study species and > 8 months in *B. cirrhosis*; Munehara 1991). Rose bitterling embryos

440   developing on the gills of freshwater mussels are known to gain nutrients and oxygen from

441   the water taken in by the mussels (Spence and Smith 2013). However, this may not be the

442   case in sculpins, because their eggs are placed where few or no nutrients may be available due

443   to the filtered water, and larvae are likely to leave their host soon after hatching since they are

444   capable of active swimming (Kimura et al. 1987, 1988; Awata S. pers. obs.).

445   Second, eggs are likely protected from predators by being hidden inside the hosts

446   (Hunter 1969; Munehara 1991). Since eggs are completely hidden in solitary tunicates that

447   have rigid bodies, predation avoidance may be highly effective. Furthermore, many species of

448   sponges are chemically defended from predation by marine animals, such as fishes, sea

449   urchins, sea stars and hermit crabs (e.g. reviewed in Paul and Puglisi 2004; Paul et al. 2006).

450   Thus, oviposition in sponges may also be effective for predation avoidance due to their

451   chemical defences. Third, females are not required to provide parental care, which may allow

452   them to utilise their energy reserves for further reproduction, such as increasing egg numbers

453   and size.

454   However, it is costly to be ostracophilous. First, the development of elongated

455   ovipositors and unique spawning behaviour are essential. Second, during the spawning

456   season, sculpin females must seek out suitable, limited oviposition hosts, which might

457   increase the probability of predation and consume energy reserves. Despite such costs, the

458   benefits for female sculpins specialising in the use of tunicates and sponges outweigh the

459   costs, which may be the evolutionary forces that produce and maintain the unique spawning

460   behaviours observed in sculpins. This also may be the case for other ostracophilous fishes

461   such as bitterlings, snailfishes and Japanese tubesnouts (Leung 2014).

462   **Costs and benefits for hosts**

463   In contrast to the potential benefits obtained by the sculpins, it is unlikely that sculpins and

464   their eggs have positive impacts on the host tunicates and sponges. Conversely, the eggs

465   deposited by sculpins may adversely affect their hosts. One possible cost for the hosts

466   harbouring sculpin egg masses is the reduction of sea water exchange, as observed in mussels

467   (Smith et al. 2001; Mills and Reynolds 2002, 2003; Kitamura 2005). In many cases, one egg

468   mass was found in a solitary tunicate, and occupied only a small space in the atrium.

469   However, the eggs, especially those oviposited by larger sculpin species such as

470   *Pseudoblennius* sp. "Kirin-anahaze", sometimes entirely fill up the tunicate atrium due to

471   their large clutch size (see Table S3). In this case, the hosts cannot effectively exchange

472   seawater and consequently might incur some costs such as lowered growth and survival from

473   the overload of fish eggs. Such an overload of fish eggs has been reported for the snailfish-

474   crab system, where the load of the egg masses in the gill cavity of crabs deposited by snail

475   fish caused severe damage to their gills (Love and Shirley 1993; Somerton and Donaldson

476   1998; Poltev and Mukhametov 2009). Spence and Smith (2013) have also found that some

477   host mussels with many bitterling eggs died, probably due to severe competition for oxygen

478   between the host mussels and bitterling embryos. Since sculpin eggs are not oviposited on the

479   gills of tunicates through the incurrent siphon, sculpin eggs unlikely do cause severe damage

480   to the respiratory system of sponges and tunicates. Experimental work is needed to ascertain

481   the cost to the hosts of sculpin eggs.

482   **Conclusions**

483   In summary, we have shown host selection and variation of ovipositor length in eight out of

484   nine sympatric marine sculpins found off the shore of an island in the Sea of Japan. To date,

485   ostracophilous behaviour has only been described for a small number of fish species, and

486   therefore only limited data on the behavioural and morphological adaptations of parasites and

487   hosts are available, with the exception of the bitterlings. A more recent study, however, has

488   found evidence of ostracophilous behaviour in the tubenose poacher *Pallasina barbata*

489   (Momota and Munehara 2017). Sculpins and tubenose poachers are different fish groups from

490   other ostracophilous fishes (Leung 2014), suggesting that ostracophilous reproductive strategy

491   has likely evolved in parallel across different taxa (Leung 2014). This study has shown the

492   morphological adaptations of sculpin females at the interspecific level. However, ongoing

493   research will need to examine the intraspecific variation in ovipositor size and morphology,

494   depending on the host species used and their size. Studying ostracophils at the population

495   level might be particularly worthwhile to identify the (co-)evolution of behavioural and

496   morphological adaptations of parasites and their hosts.

Ethical statement

Acknowledgements

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Figure legends

Fig. 1 Location of sampling sites. (a) Sado Island located 45 km north west of Niigata, Japan. We collected sculpins that oviposit on rock surface at Usujiri, Hokkaido, Japan. (b) Four sampling sites on the coast of the island. (c) Kitakoura Port. Monthly collection of tunicates was conducted along the south breakwater of the port (indicated by an *ellipse*).

Fig. 2 Examples of photographs of eggs deposited in sponges and tunicates. (a) Egg masses deposited by females of *Pseudoblennius marmoratus* in sponges. (b) Eyed eggs of *Vellitor centropomus* in a colonial tunicate. (c) Colonial tunicate; at least five egg masses were deposited in this colony. Colony width: c.a. 100 mm. (d) *Halocynthia ritteri*, the solitary tunicates most frequently used by sculpins. (e) Underwater photograph of *H. roretzi*. (f) Eyed eggs of *Ps. cottoides* found in *H. ritteri*. (g) Eyed eggs of *Pseudoblennius* sp. "Kirin-anahaze" deposited in *H. roretzi*. (f) Eyed *Furcina osimae* eggs in *Pyura sacciformis*. Scale bars indicated in (a), (b), (f), (g) and (h): 10 mm.

Fig. 3 Size frequency distribution of solitary tunicates collected during study periods. (a) *Microcosmus hartmeyeri*, (b) *Herdmania japonica*, (c) *Halocynthia roretzi*, (d) *Halocynthia ritteri*, (e) *Polycarpa cryptocarpa kroboja*, (f) *Pyura sacciformis* and (g) *Cnemidocarpa irene*. Panels are arranged in decreasing order of the average long diameter (LD) of tunicates. *White*, *grey* and *black bars* indicate tunicates with sculpin egg masses, with Japanese tubesnout egg masses and without any egg masses, respectively. n_{sc}: number of tunicates with sculpin eggs, n_{tu}: number of tunicates with eggs of Japanese tubesnout (the details of which will be published in a separate paper) and n_{ne}: number of tunicates with no fish eggs.

665 **Fig. 4** (a) Ovipositor length (OL) increased with standard length (SL) of sculpins and differed

666 among sculpins that utilised four different types of hosts. *Circles*, *triangles* and *squares*

667 denote the species that used solitary tunicates, colonial tunicates and sponges, and rock

668 surfaces as deposition sites, respectively. Regression lines for different types of hosts are from

669 the reduced model of ANCOVA. Relative OL were statistically significantly different between

670 all combinations of the four types (Tukey's HSD, $p < 0.05$). (b)–(e) Examples of photographs

671 of ovipositors (indicated by the arrows) in the sculpins of the four types. (b) *Pseudoblennius*

672 *cottoides*; (c) *Vellitor centropomus*; (d) *Ps. marmoratus*; and (e) *Alcichthys alcicornis*. Scale

673 bars: 10 mm.

674 **Fig. 5** Body size and ovipositor length of parasitic sculpins were closely related to the size of

675 solitary tunicates selected by each sculpin species as spawning hosts. (a) Female standard

676 length (SL) and (b) ovipositor length (OL) of six species of sculpins that used solitary

677 tunicates as hosts. (c) Long diameter (LD) and (d) egg mass position of tunicates utilised as

678 hosts. Means (± SD) with different letters are significantly different (Tukey's HSD, $p < 0.05$).

679 Sample sizes are indicated as numerals in bars. *ND*, no data. Bars are arranged in decreasing

680 order of the average SL of sculpins from left to right.

681

Supporting information

Figure S1 Maximum likelihood phylogeny of nine species of potentially ostracophilous sculpins, with *Jordania zonope* as an outgroup, as reconstructed by MEGA ver. 7.0.14. using 800 bp *cytb* sequences, with the settings of the Tamura Nei model and 1000 bootstrap replicates. Values at nodes are bootstrap percentages.

Figure S2 Maximum likelihood phylogeny of the potential parental species and 205 eggs from 113 egg masses as reconstructed by MEGA ver. 7.0.14. using 800 bp *cytb* sequences, with the settings of the Tamura Nei model and 1000 bootstrap replicates. Values at nodes are bootstrap percentages.

Figure S3 Maximum likelihood phylogeny of the potential parental species and 18 eggs from 14 egg masses as reconstructed by MEGA ver. 7.0.14., using 628 bp *cytb* sequences, with the settings of Tamura Nei model and 1000 bootstrap replicates. Values at nodes are bootstrap percentages.

Movie S1 Spawning behaviours of female *Pseudoblennius cottoides* in the aquarium. Six *Halocynthia ritteri* on wire-netting were placed in a 75 × 45 × 45 cm aquarium. Eggs were deposited in the atrium of a tunicate through the atrial syphon using extremely long ovipositors. Eggs were probably released into the atrium when the female opened her mouth. The female stayed still on the tunicate after spawning. She ejected her ovipositor 1 min 22 sec and left the tunicate 2 min 34 sec after the insert, respectively (not shown). This movie was filmed by H. S. on Feb. 7, 2015. Fish and tunicates were collected on the coast of Sado Island in the Sea of Japan.

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703 **Movie S2** Failure of spawning by female *Pseudoblennius cottoides* in the aquarium. The atrial

704 siphon of the tunicate closed before the sculpin female inserted her ovipositor. Settings were

705 the same as in Movie S1. This movie was filmed by H. S. on Feb. 1, 2015.

706 **Movie S3** Spawning behaviours of female *Pseudoblennius marmoratus* in the field. Eggs

707 were deposited in the spongocoel of a sponge using short ovipositors. This movie was filmed

708 by Tomonobu Uryu on Dec. 28, 2009 at Izu Oceanic Park, Izu Peninsula, Pacific coast of

709 Japan. Water temperature was about 16 °C and water depth was about 5 m.