

This is an Accepted Manuscript of an article published by Canadian Science Publishing in *Canadian Journal of Zoology*, available online: <https://doi.org/10.1139/cjz-2022-0033>.

1 **Protogynous hermaphroditism in Crustacea: a new example from Tanaidacea**

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14 **Competing interests:** The authors declare there are no competing interests.

15 **Funding:** This research was supported in part by KAKENHI grants (JP19K06800 and

16 JP20J40122) from the Japan Society for the Promotion of Science (JSPS) and by funding

17 from the Fujiwara Natural History Public Interest Incorporated Foundation (FY2015).

18 **Data availability:** The following information was supplied regarding data availability: The
19 raw data (sampling locality; sampling date) are included in the “Materials and methods”
20 section in the manuscript.

21

22 **Abstract:** Hermaphroditism has been widely reported in the Crustacea, but protogyny, in
23 which females change to males, is apparently rare and restricted to the peracarid orders
24 Isopoda and Tanaidacea. In the latter, protogyny has been demonstrated in only seven species
25 representing six genera by rearing experiments and indicated for several other genera and
26 species through morphology. Here we show, through rearing experiment and histological
27 observations, protogynous hermaphroditism in the tanaidacean species *Nesotanaïs* sp. aff.
28 *ryukyuensis* Kakui et al., 2010. Our rearing experiment and size distribution data for wild
29 individuals showed that females can change sex at various sizes (= ages). We found one
30 individual identified externally as a female containing both ovaries and testes, indicating that
31 overt female individuals undergo a short transitional phase as simultaneous hermaphrodites
32 before the sex-change molt. We discuss the relationship among the wide size distribution of
33 sex-changing females, the short life span of males, and the tube-dwelling mode of life.

34

35 Key words: Malacostraca, *Nesotanaïs* sp. aff. *ryukyuensis*, Pancrustacea, Peracarida,
36 sequential hermaphroditism, sex change, tanaids

37

38 Introduction

39 The Crustacea is an extremely diverse group not only in terms of size (more than
40 67,000 extant species; cf. Ahyong et al. 2011) but also in the diversity of sexual systems,
41 which include gonochoristic, hermaphroditic, asexual, and mixed systems (Benvenuto and
42 Weeks 2020). There are many hermaphroditic crustaceans, among which the least common
43 are protogynous hermaphrodites, which change sex from female to male (Vogt 2020).
44 Currently, protogyny has been suspected or documented in only about 20 crustacean species,
45 all of which belong to the peracarid orders Isopoda and Tanaidacea (Benvenuto and Weeks
46 2020).

47 Protogyny in the Tanaidacea, a group of small benthic crustaceans with around 1500
48 described species (Anderson 2020), has previously been demonstrated through rearing
49 experiments for six species in Leptocheliidae and one species in Nototanaididae (Table 1); in
50 two of these species, *Heterotanais oerstedii* (Krøyer, 1842) and *Nototanais dimorphus*
51 (Beddard, 1886) (Bückle-Ramírez 1965; Marinovic 1987), the internal morphology was also
52 examined. *Sinelobus stanfordi* (Richardson, 1901) (Tanaididae) and *Neotanais micromopher*
53 Gardiner, 1975 (Neotanaididae) were judged to be protogynous on the basis of external and
54 internal morphology and the size distribution of developmental stages (Lang 1958; Gardiner
55 1975). *Monokalliapseudes* (Kalliapseudidae) and *Nototanoides* (Nototanaididae) may also
56 contain protogynous members, because male polymorphism and/or a female-skewed sex ratio

57 was observed in some species (Pennafirme and Soares-Gomes 2009; Kakui and Yamasaki
58 2013; cf. Bückle-Ramírez 1965).

59 Bückle-Ramírez (1965) showed that all males in *H. oerstedii* have (1) degenerate
60 mouthparts and (2) pronounced sexual dimorphism in the chelipeds. These features are also
61 evident in males of the other protogynous species listed in Table 1. This combination of
62 characters has been observed in many genera for which the sexual system has not been
63 studied, suggesting that additional protogynous tanaidacean species remain to be discovered.
64 In this study, we investigated the sexual system in one such species in *Nesotanais*
65 (*Nototanaidae*), through a rearing experiment and histological observations.

66

67

68 **Materials and methods**

69 **Field sampling**

70 Samples were collected from the brackish downstream region of the Oura River
71 (26°33'36.7"N 128°02'36.0"E) on Okinawa Island, Japan on 9 March 2014, 29 July 2014, and
72 12 January 2016. Mud sediment containing tanaidaceans was collected along a mangrove
73 forest and filtered through 0.42 mm mesh; tanaidaceans were picked from the residue. Except
74 for those used in a rearing experiment, individuals were fixed in Bouin's fluid or 70%
75 ethanol.

76

77 **Taxonomy**

78 Our study species (Figure 1) morphologically resembled *Nesotanaïs ryukyuensis*
79 Kakui et al., 2010 described from Noha River on Okinawa Island (cf. Kakui et al. 2010), but
80 showed a large genetic distance from *N. ryukyuensis* topotypes (ca. 11% Kimura (1980) 2-
81 parameter distance between their 18S rRNA sequences; KK unpublished data); we thus refer
82 to our study species as *Nesotanaïs* sp. aff. *ryukyuensis* Kakui et al., 2010.

83

84 **Staging, sexing, and measurements**

85 We categorized specimens into one of two developmental stages: (1) “manca,” sex-
86 indeterminate, post-embryonic juveniles in which pereopods 6 and pleopods were lacking or
87 incompletely developed; and (2) “post-manca,” individuals with fully developed pereopods 6
88 and pleopods. Post-mancae with paired genital cones on the ventral surface of pereonite 6
89 were regarded as males, and those lacking the cones as females (see Supplementary Figure
90 S1¹). We measured the carapace length (CL; mm) as an index of body size for 38 males
91 (including eight reared individuals), 66 females (including two reared individuals), six
92 exuviae shed by females when they changed sex, and two mancae. CL was correlated with
93 body length (BL; mm) (for males, $BL = 2.96CL + 0.29$, $R^2 = 0.66$, $n = 17$; for females, $BL =$
94 $4.45CL - 0.13$, $R^2 = 0.85$, $n = 38$; cf. Supplementary Figure S2¹).

¹Supplementary figures are available with the article at <https://doi.org/10.1139/cjz-XXXXXXXXXXXX>.

95

96 Rearing experiment

97 Nineteen females lacking a marsupium (= brood pouch) and five males were isolated
98 in brackish water (3‰ salinity), each in one well of a 6-well cell-culture plate, at 20°C in the
99 dark, fed every 2–4 days with porphyzized dry feed for crayfish (JAN code 4971618829092;
100 Kyorin), and observed every 1–4 days (but see the Results section). The experimental periods
101 were 46–65 days. Exuviae successfully recovered were preserved in 70% ethanol. Individuals
102 were fixed in Bouin's fluid.

103

104 Histology

105 Whole body (except cephalothorax and cephalothoracic appendages) of four females
106 and five males fixed in Bouin's fluid was used for histology (Table 2). Paraffin sections 5 µm
107 thick were prepared, stained with Mayer's hematoxylin and eosin (HE) using standard
108 techniques, and observed with an Olympus BX53 light microscope.

109

110

111 Results**112 Rearing experiment**

113 The results of rearing experiment are summarized in Table 3. Seven of 19 females

114 changed sex. They changed sex by 36 days subsequent to isolation. Prior to the sex-changing
115 molt, up to two molts were observed. One female survived as female for more than 65 days
116 subsequent to isolation.

117 No molts by males (including seven sex-changed ones) were observed. One male
118 survived for more than 44 days after sex change.

119

120 **Size distribution**

121 The ranges in CL were 0.17–0.19 mm for mancae ($n = 2$), 0.23–0.43 mm for females
122 ($n = 66$), and 0.28–0.49 mm for males ($n = 38$); that for females with a marsupium ($n = 15$)
123 was 0.32–0.43 mm (Figure 2). During the rearing experiment, six exuviae that females shed
124 when they changed sex were recovered. The CLs for the six exuviae and the corresponding
125 sex-changed male for each were as follows (in mm): 0.23 and 0.28; 0.33 and 0.38; 0.35 and
126 0.42; 0.36 and 0.41; 0.37 and 0.41; and 0.38 and 0.44 (connected by lines in Figure 2).

127

128 **Histology**

129 Three females (Specimens F1, F2, and F4; Figure 3A, D) had paired ovaries, with
130 dense yolk granules in the oocytes. One female (Specimen F3; Figure 3B, E) had paired
131 ovaries and testes. Male gonopores were not observed. Yolk granules in the oocytes were
132 sparse.

133 Five males, including one sex-changed individual (Specimens M1–4 and FM1; Figure
134 3C, F) had paired testes. Male gonopores were observed on the ventral surface of pereonite 6.
135 The digestive organs (gut and paired hepatopancreases) were shrunken, and the gut was
136 blackened (Figures 1B, 3F).

137

138

139 **Discussion**

140 **Sexual system in *Nesotanaïs* sp. aff. *ryukyuensis* and other protogynous tanaidaceans**

141 Our rearing experiment showed that females (although maybe not all females; see
142 below) of *Nesotanaïs* sp. aff. *ryukyuensis* can change sex at various sizes. Seven females that
143 changed sex ranged from 0.23 mm to 0.38 mm in CL; their CL increased by an average of
144 0.06 mm (range 0.04–0.07 mm) after sex change (Figure 2). In males, the digestive system
145 (e.g., mouthparts, gut, and hepatopancreases) had degenerated and no molts were observed,
146 suggesting that in this species, males must be terminal, devoted to reproduction without
147 feeding or molting. Wild males showed large variation (0.29–0.49 mm) in CL, indicating that
148 females of *N.* sp. aff. *ryukyuensis* change sex at various sizes, even in the wild.

149 Twelve females, one of which survived for more than two months, did not change sex
150 during our experimental period. This indicates that, as suggested for *H. oerstedii* (Bückle-
151 Ramírez 1965), *N.* sp. aff. *ryukyuensis* likely contains females that can change sex

152 (“hermaphrodites”) as well as those that cannot (“gonochoristic females”).

153 We found one female bearing paired ovaries and testes (Specimen F3; Figure 3E). Its
154 ovary may have been degenerating, as yolk granules were sparse in the oocytes. An
155 individual bearing both ovaries and testes has also been reported in the confamilial
156 protogynous species *Nototanaïs dimorphus*, but its overt sex was not stated (Marinovic
157 1987). Bückle-Ramírez (1965) observed nearly all females of *H. oerstedii* to change body
158 color from yellowish brown to bluish at about two days (on average) before the sex-change
159 molt, and that the bluish females bore paired gonads containing sperm. Although it is
160 unknown whether our Specimen F3 eventually molted and changed sex, our and previous
161 observations suggest that females of protogynous leptocheliid and nototanaid species may,
162 after a short simultaneously hermaphroditic, transitional phase in which degenerating ovaries
163 and developing testes coexist (the condition in Specimen F3), enter a phase bearing only
164 testes (the condition in bluish females of *H. oerstedii*), and then molt to complete the sex
165 change. It should be noted that although Lang (1958) reported individuals bearing both
166 ovaries and testes in *Sinelobus stanfordi*, the structure he identified (Lang 1958: plate II,
167 figure 2) as an ovary was instead thoracic gland 1 *sensu* Kaji et al. (2016), because it had a
168 thick duct.

169 It is not known what factors induce the sex change in *N. sp. aff. ryukyuensis*, but the
170 social structure (presence or absence of males) is a possible factor. In the protogynous

171 leptocheiliid species *H. oerstedii* and *Chondrochelia dubia* (Krøyer, 1842), the frequency of
172 sex change was higher in aquaria containing only females than in aquaria containing both
173 sexes, indicating that the social structure affects whether females change sex (Bücker-
174 Ramírez 1965; Highsmith 1983). Having observed that a female in an aquarium where
175 freshly ground males were added every two days had not changed sex, Bücker-Ramírez
176 (1965) speculated that the sex-change inhibiting substance may be a male pheromone. After
177 observing strong seasonality in reproduction in Antarctic *N. dimorphus*, Marinovic (1987)
178 suggested that photoperiod may also affect whether females change sex.

179 Bücker-Ramírez (1965) classified males of *H. oerstedii* into two types: (1) “primary
180 males,” which emerge from a “neuter” individual, that is, a female-like post-manca lacking
181 internal sexual features; (2) “secondary males,” which develop by sex change from a female.
182 Other researchers have followed his classification. Given that males in gonochoristic species
183 already bear genital cones at the first post-manca instar (cf. Messing 1983; Toniollo and
184 Masunari 2007), however, female-like neuter individuals *sensu* Bücker-Ramírez (1965) might
185 be females, which indicates that the difference between primary and secondary males merely
186 corresponds to a difference in the female ontogenetic stage at sex change (i.e., all males
187 develop by sex change from a female). In summary, protogynous tanaidacean species, or at
188 least *H. oerstedii* and *N. sp. aff. ryukyuensis*, may consist of (1) gonochoristic females, (2)
189 hermaphrodites before sex-change, and (3) sex-changed hermaphrodites (= males).

190

191 **Why does sex change occur at various sizes in protogynous tanaidaceans?**

192 That females can change sex at various sizes seems to be a general phenomenon in
193 protogynous tanaidacean species. As with *N. sp. aff. ryukyuensis*, captive individuals of *H.*
194 *oerstedii* can change sex from female to male at various sizes (Bückle-Ramírez 1965), and a
195 wide size range in wild males has been observed in all protogynous species (Table 1) except
196 for *Chondrochelia neapolitana* (Sars, 1882), for which this information is lacking
197 (Jazdzewski 1969; Highsmith 1983; Ishimaru 1984, 1985; Stoner 1986; Marinovic 1987;
198 Modlin and Harris 1989).

199 Larger males of protogynous species may be superior to smaller males in
200 reproduction. All protogynous tanaidaceans are tube-dwellers (Stoner 1986; Kakui 2021).
201 While limited data are available on male-male interactions, Highsmith (1983) showed that
202 larger males of *C. dubia* are superior to smaller males in male-male fighting. In *Nesotanaeis*
203 males, the large, shield-like chelae (Figure 1C, D) bearing a presumptive stridulatory organ
204 (Figure 1E; Kakui et al. 2010) may function to block intruders during precopulatory guarding
205 in the tube; larger chelae may be more effective in guarding.

206 Males in protogynous tanaidaceans probably have a short life span. As Highsmith
207 (1983) pointed out, a marked difference between protogynous tanaidaceans and other
208 protogynous animals (e.g., fishes and isopods) is that the digestive system in males of the

¹Supplementary figures are available with the article at <https://doi.org/10.1139/cjz-XXXXXXXXXXXX>.

209 former is not functional, meaning that males live only for a short period in a population. In
210 addition, possibly because males leave their tube and search the bottom for tube-dwelling
211 females for copulation, males of tube-dwelling tanaidacean species have a higher predation
212 risk than females (Mendoza 1982; Marinovic 1987; Kakui 2015). Furthermore, Kakui (2015)
213 suggested that large males may have higher predation risk than small males.

214 Male tanaidaceans can copulate with multiple females during their short life span;
215 they visit the tube of a preparatory female (= female with developing oostegites), engage in
216 copulation after a short precopulatory-guarding period, and then leave the tube to find another
217 female (Bückle-Ramírez 1965; Johnson and Attramadal 1982). Female tanaidaceans can
218 produce up to two or three broods of eggs if they spend whole their life as females (cf.
219 Bückle-Ramírez 1965); between broods, they lose the brood pouch and form a new one via
220 several molts.

221 Since protogynous tanaidacans are tube dwelling and have narrow home ranges
222 (females narrower than males), this would make it advantageous for females to sex change to
223 males whenever there were not males around to fertilize them. This system would work best
224 if females could change sex at any size. A single female changing sex in a local area would
225 negate the necessity for other females to do so, since a male can fertilize multiple females, so
226 the system is flexible, sort of a "supply upon demand" system. Overall reproductive success
227 of the population would be maximized if only as many females as absolutely necessary

228 changed sex, and if females were able to put off changing sex as long as possible, because the
229 overall reproductive output of small females laying only a single brood is less than that of
230 females that can grow larger and produce multiple broods.

231 In protogynous tanaidaceans, males have shorter life spans than females as a direct
232 consequence of their not being able to feed, although this begs the question why they lose the
233 digestive tract upon sex change. This could be an ontogenetic consequence of the sex change
234 itself—if, for example, the sex change evolved in such a way that it could be accomplished
235 developmentally only with loss of the digestive system. However, since males must disperse
236 relatively widely from their tubes to find females, their life span will be shorter than that of
237 females as a simple consequence of greater mortality through predation. In terms of
238 reproduction, it may thus have been evolutionarily advantageous for males to devote all their
239 effort to seeking females rather than partly to seeking food, given that they might not survive
240 very long anyway due to predation. If this were the case, the digestive would have been lost
241 because it was no longer needed, rather than as an unavoidable developmental correlate of the
242 sex change.

243

244

245 **Conclusions**

246 This study revealed that *Nesotanaeis* sp. aff. *ryukyuensis* is a protogynous species.
247 Future research involving rearing experiments will discover further examples of protogynous
248 tanaidaceans, especially in groups whose males bear degenerate mouthparts. Reports of
249 protogyny are still few in Crustacea and our knowledge on the sexual system is not sufficient.
250 More studies on protogynous crustaceans will be necessary to understand how greatly diverse
251 crustacean sexual systems have evolved.

252

253

254 **Acknowledgments**

255 We thank Hiroshi Yamasaki for help in field sampling; Shinri Tomioka for help in
256 rearing tanaidaceans; and Matthew H Dick for reviewing the manuscript and editing the
257 English. This study was supported in part by KAKENHI grants (JP19K06800 and
258 JP20J40122) from the Japan Society for the Promotion of Science (JSPS) and by funding
259 from the Fujiwara Natural History Public Interest Incorporated Foundation (FY2015).

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- 332

333 **Figure legends**

334 **Figure 1.** *Nesotanaïs* sp. aff. *ryukyuensis*. (A, B) Live animals, dorsal view; (A) female; (B)
335 male. (C–E) SEM images of left male cheliped; C, outer view; D, anterior view of chela; E,
336 inner view of chela. Abbreviations: fl, flange; gu, gut; ov, ovary. Scale bars: A, B, 0.5 mm;
337 C–E, 0.1 mm.

338

339 **Figure 2.** Size distribution of female and male individuals of *Nesotanaïs* sp. aff. *ryukyuensis*.
340 Gray-filled circles, females with marsupium; open circles, individuals lacking marsupium.
341 Circles derived from same individual (i.e., a sex-changed individual and the exuvia that the
342 individual shed when it changed sex) are connected with a line.

343

344 **Figure 3.** *Nesotanaïs* sp. aff. *ryukyuensis*, fixed individuals. (A–C) Habitus, lateral view. (D–
345 F) Cross-sections stained with HE. (A, D) Specimen F1. (B, E) Specimen F3. (C, F)
346 Specimen FM1. Abbreviations: d, dorsal; gu, gut; hp, hepatopancreas; ov, ovary; te, testis; v,
347 ventral; yg, yolk granule. Scale bars: A–C, 0.5 mm; D–F, 0.05 mm.

1 **Table 1.** Tanaidacean species in which protogyny has been demonstrated by rearing
2 experiments.

Family	Species	Selected sources
Leptocheliidae	<i>Chondrochelia dubia</i>	Highsmith (1983); Stoner (1986)
	<i>Chondrochelia neapolitana</i>	Ishimaru (1984)
	<i>Hargeria rapax</i>	Modlin and Harris (1989)
	<i>Heterotanais oerstedii</i>	Forsman (1956); Bückle-Ramírez (1965); Jażdżewski (1969)
	<i>Leptochelia forresti</i>	Stoner (1986)
	<i>Makassaritanais itoi</i>	Ishimaru (1985)
Nototanaidae	<i>Nototanaais dimorphus</i>	Marinovic (1987)
	<i>Nesotanaais</i> sp. aff. <i>ryukyuensis</i>	This study

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5 **Table 2.** Details of specimens used for histology. * carapace length (CL) of exuvia.

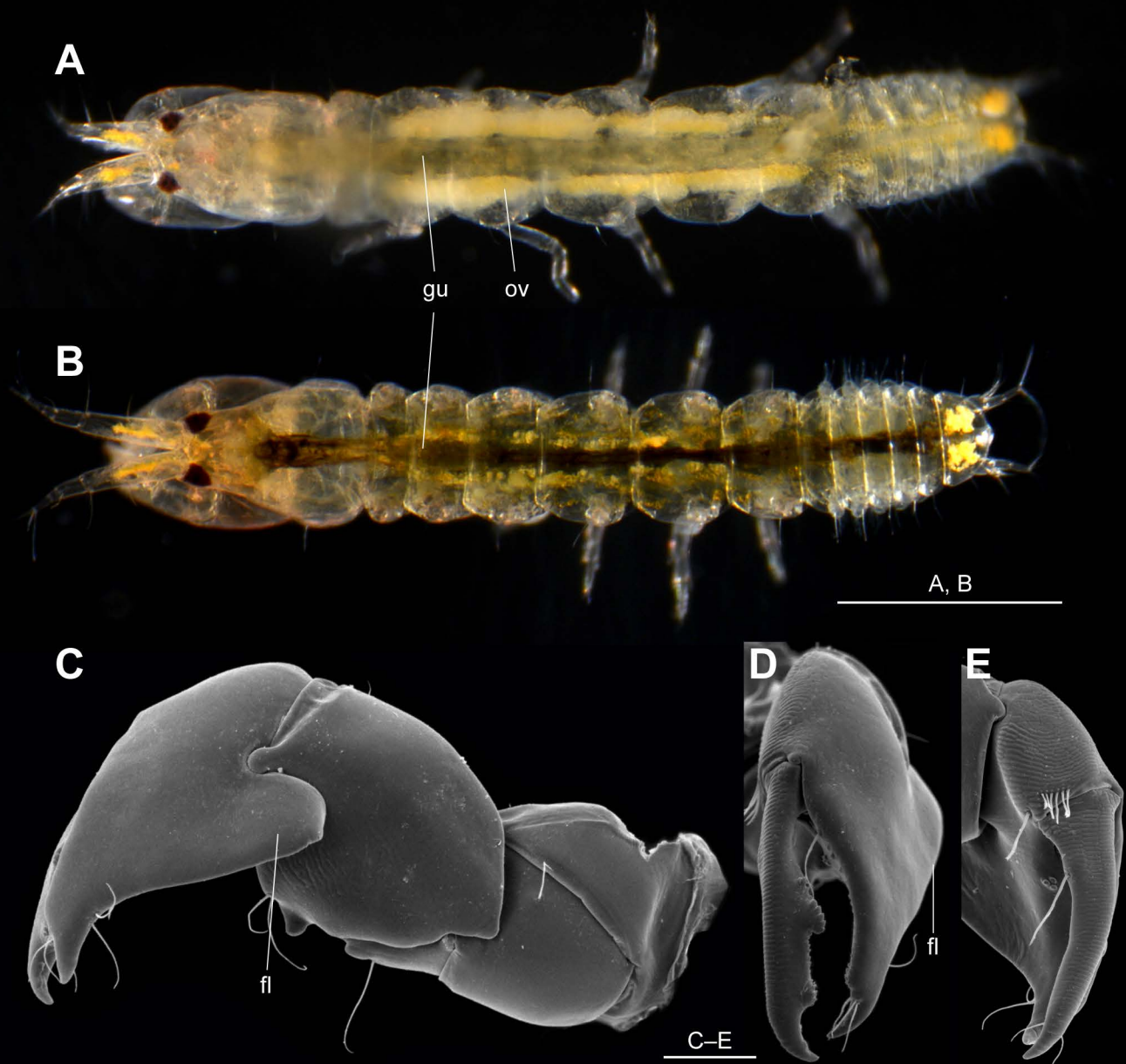
Specimens	Sex in appearance	CL (mm)	Sampling date and remarks
F1	Female	0.34	9 Mar 2014; bearing empty marsupium
F2	Female	0.34	9 Mar 2014
F3	Female	0.35	9 Mar 2014
F4	Female	0.38	9 Mar 2014; used in rearing experiment
M1	Male	0.29	29 Jul 2014
M2	Male	0.30	29 Jul 2014
M3	Male	0.34	29 Jul 2014
M4	Male	0.38	29 Jul 2014
FM1	Male (sex changed)	0.43 (0.38*)	9 Mar 2014; used in rearing experiment; sex-changed from female to male during experiment

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8 **Table 3.** Summary of rearing experiment. Abbreviations: das, days after sex change; dsi, days subsequent to isolation; +, sex changed; -, sex not
 9 changed

Sampling date	Isolation date	Individual	Sex change	Death date	Remarks
9.iii.2014	11.iii.2014	3 females	–	before 16 dsi	First observation at 16 dsi
		1 female	+; date unknown (before 16 dsi)	before 16 dsi	First observation at 16 dsi
		1 female	+; date unknown (before 16 dsi)	60 dsi, >44 das	First observation at 16 dsi
		1 female	+; 34 dsi	(fixed at 65 dsi, 31 das)	2 molts observed prior to sex-changing molt; Specimen FM1
		1 female	–	(fixed at 65 dsi)	2 molts observed prior to fixation; Specimen F4
	30.iii.2014	2 males	–	before 16 dsi	First observation at 16 dsi
		1 female	+; date unknown (before 27 dsi)	(fixed at 46 dsi, >19 das)	
		1 female	+; 32 dsi	(fixed at 46 dsi, 14 das)	1 molt observed prior to sex-changing molt
		1 female	–	(fixed at 46 dsi)	1 molt observed prior to fixation
		2 males	–	7–25 dsi	
12.i.2016	15.i.2016	1 female	+; 3 dsi	42 dsi, 39 das	No molt observed prior to sex-changing molt
		1 female	+; 36 dsi	(fixed at 36 dsi, 0 das)	2 molts observed prior to sex-changing molt
		7 females	–	10–51 dsi	0–3 molts observed prior to death
		1 male	–	26 dsi	



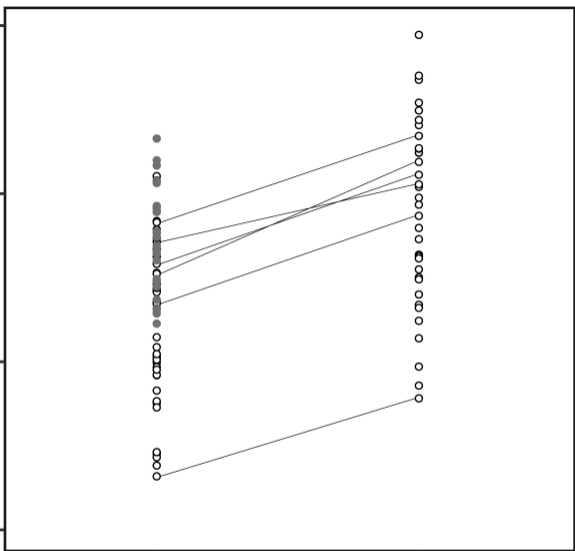
Carapace length (CL; mm)

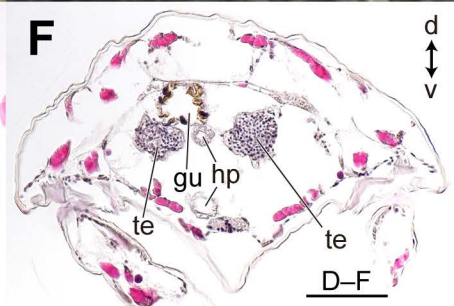
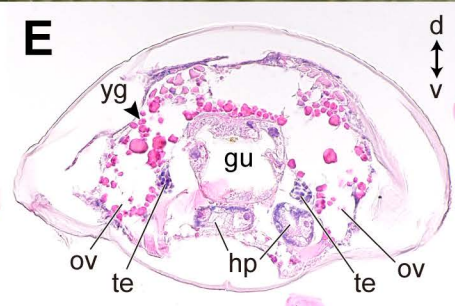
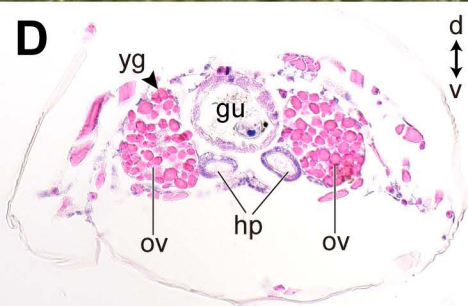
0.5
0.4
0.3
0.2

Female

Male

Sex





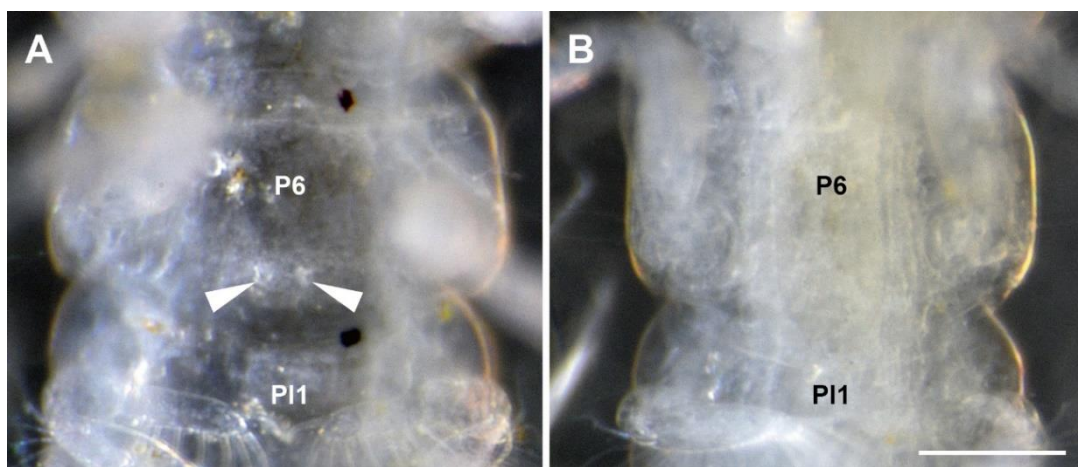
Journal name: Canadian Journal of Zoology

Article title: Protogynous hermaphroditism in Crustacea: a new example from Tanaidacea

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Supplementary Figure S1. Ventral view of pereonite 6 and pleonite 1 of *Nesotanaïs* sp. aff. *ryukyuensis*. (A) Male with a pair of genital cones (arrowheads). (B) Female without genital cones. Abbreviations: P6, pereonite 6; P11, pleonite 1. Scale bar: 0.1 mm.



Supplementary Figure S2. Relationship between body length (BL) and carapace length (CL) in *Nesotanaïs* sp. aff. *ryukyuensis*.

