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Author(s)	Kakui, Keiichi; Hiruta, Chizue
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Protogynous hermaphroditism in Crustacea: a new example from Tanaidacea

Keiichi Kakui and Chizue Hiruta

Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo, Hokkaido, Japan

Corresponding author. Keiichi Kakui. E-mail: kakui@eis.hokudai.ac.jp

ORCID iD

Keiichi Kakui: 0000-0003-4630-9065

Chizue Hiruta: 0000-0002-0194-6573

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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

[1]Supplementary figures are available with the article at https://doi.org/10.1139/cjz-XXXXXXXXXXX.

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

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

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Introduction

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

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

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

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

Materials and methods

Field sampling

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

[1]Supplementary figures are available with the article at https://doi.org/10.1139/cjz-XXXXXXXXXXX.

76

77 **Taxonomy**

78 Our study species (Figure 1) morphologically resembled *Nesotanais 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 *Nesotanais* 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 S1[1]). 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 S2[1]).

[1]Supplementary figures are available with the article at https://doi.org/10.1139/cjz-XXXXXXXXXXX.

Rearing experiment

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

Histology

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

Results

Rearing experiment

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

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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.

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

Discussion

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

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

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

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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 *Nototanais 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

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

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

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

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

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

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

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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

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

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

Conclusions

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

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Figure legends

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

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

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

[1]Supplementary figures are available with the article at https://doi.org/10.1139/cjz-XXXXXXXXXXX.

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

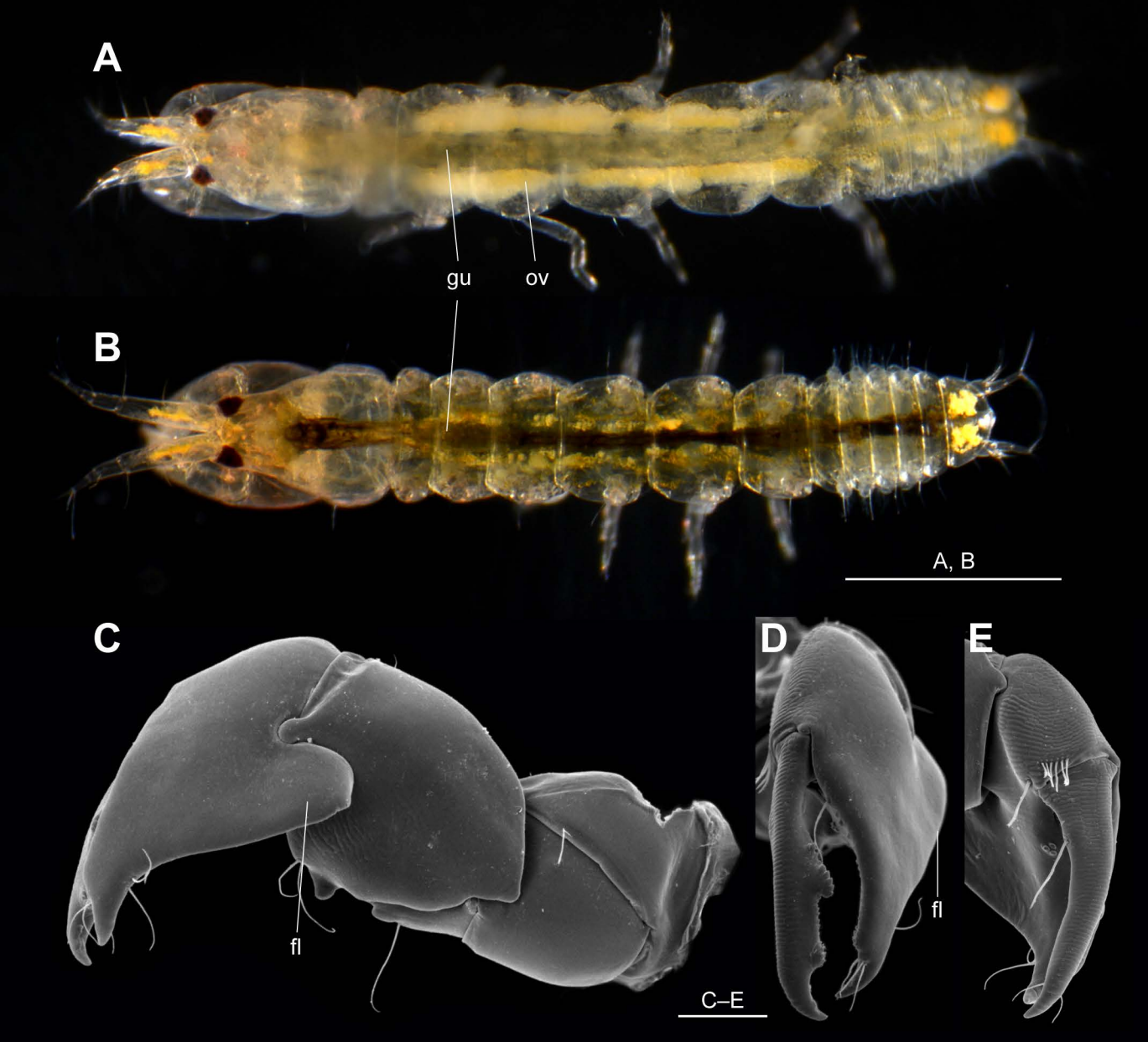
Family	Species	Selected sources
Leptocheliidae	*Chondrochelia dubia*	Highsmith (1983); Stoner (1986)
	Chondrochelia neapolitana	Ishimaru (1984)
	Hargeria rapax	Modlin and Harris (1989)
	Heterotanais oerstedii	Forsman (1956); Bückle-Ramírez (1965); Jażdżewski (1969)
	Leptochelia forresti	Stoner (1986)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

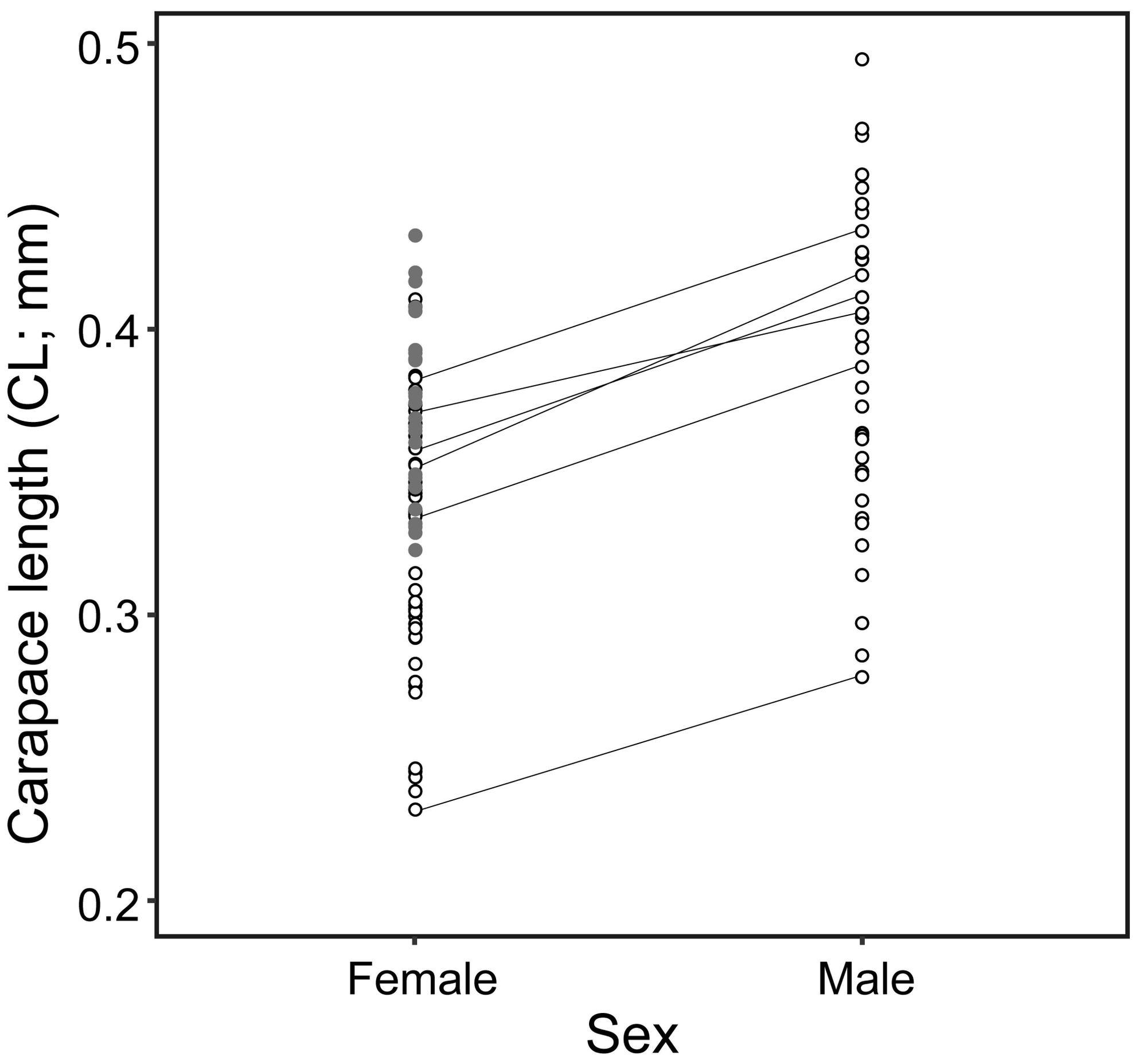
Table 2. Details of specimens used for histology. * carapace length (CL) of exuvia.

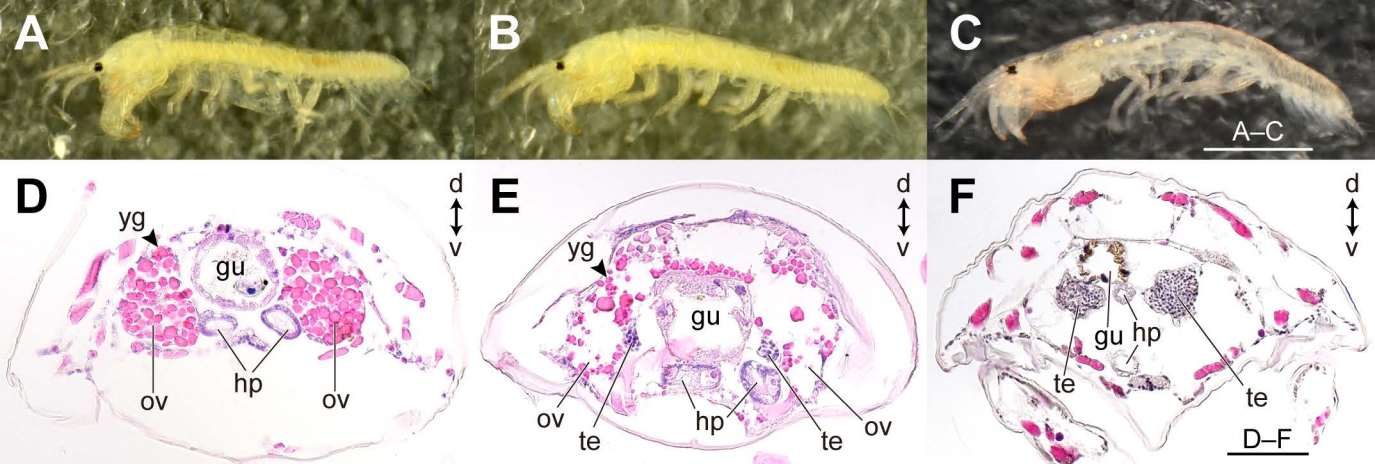
Specimens	Sex in appearance	CL (mm)	Sampling date and remarks
F1	Female	0.34	9 Mar 2014; bearing empty marsupium
F2	Female	0.34	9 Mar 2014
F3	Female	0.35	9 Mar 2014
F4	Female	0.38	9 Mar 2014; used in rearing experiment
M1	Male	0.29	29 Jul 2014
M2	Male	0.30	29 Jul 2014
M3	Male	0.34	29 Jul 2014
M4	Male	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

Table 3. Summary of rearing experiment. Abbreviations: das, days after sex change; dsi, days subsequent to isolation; +, sex changed; –, sex not 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
		2 males	–	before 16 dsi	First observation at 16 dsi
	30.iii.2014	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 dis)	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	


A
gu
ov
B
A, B
C
fl
D
fl
E
C–E


0.5
0.4
0.3
0.2
Carapace length (CL; mm)
Female
Male
Sex


A
B
C
A–C
D
yg
gu
ov
hp
ov
d
v
E
yg
gu
ov
te
hp
te
ov
d
v
F
gu
hp
te
te
D–F
d
v