



Extreme secondary sexual dimorphism in the genus *Florarctus* (Heterotardigrada: Halechiniscidae)

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Abstract

Secondary sexual dimorphism in florarctin tardigrades is a well-known phenomenon. Males are usually smaller than females, and primary clavae are relatively longer in the former. A new species *Florarctus bellahelenae*, collected from subtidal coralline sand just behind the reef fringe of Long Island, Chesterfield Reefs (Pacific Ocean), exhibits extreme secondary dimorphism. Males have developed primary clavae that are much thicker and three times longer than those present in females. Furthermore, the male primary clavae have an accordion-like outer structure, whereas primary clavae are smooth in females. Other species of *Florarctus* Delamare-Deboutteville & Renaud-Mornant, 1965 inhabiting the Pacific Ocean were investigated. Males are typically smaller than females, but males of *Florarctus heimi* Delamare-Deboutteville & Renaud-Mornant, 1965 and females of *Florarctus cervinus* Renaud-Mornant, 1987 have never been recorded. The Renaud-Mornant collection was re-examined, and type series were analysed. *Florarctus heimi* and *F. cervinus* were always found together in the coralline sand of Heron Island (Great Barrier Reef). The animals were kept alive and surveyed in the laboratory of the Queensland Museum. All studied individuals of the larger *F. heimi* (up to ca. 400 µm) were females, and all adults of the smaller *F. cervinus* (about 170 µm) were males. Males of *F. cervinus* were observed mating with females of *F. heimi*. Following those morphological and behavioural lines of evidence, we propose that *F. cervinus* is a junior synonym of *F. heimi*. Based on the discovery of dimorphism in *F. bellahelenae* sp. nov. and the strong sex-related morphological disparities in *F. heimi*, we suggest that extreme secondary dimorphism may be present in other florarctin arthrotardigrades.

Keywords Clavae · Fertilisation · Mating behaviour · Meiofauna · Tardigrades

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Introduction

Meiofauna comprises microscopic animals (Schmidt-Rhaesa 2020), some of which were often referred to as “smaller neglected phyla” (e.g. Kershaw 1983; Blaxter et al. 2004). This is probably because meiofauna has been largely understudied in many aspects, such as the lack of biodiversity surveys and the unknown overall species richness (Curini-Galletti et al. 2012), through uncertain conservation needs (Roberts et al. 2002; Martínez et al. 2020), to obscure role in ecosystem functioning (Schratzberger and Ingels 2018). In tardigrades, there has been an additional bias towards studying limno-terrestrial lineages, while marine taxa have been left relatively unexplored (Bartels et al. 2016).

Unsurprisingly, mating, copulation and related sexual reproduction behaviours remain generally poorly investigated for most meiofaunal organisms. There is a single report on Kinorhyncha (Neuhaus and Higgins 2002), two studies on Gastrotricha (Ruppert 1978a, 1978b), and a few studies on

monogonont Rotifera (Gilbert 1963; Rico-Martínez and Snell 1997; Velázquez-Rojas et al. 2002; Rico-Martínez and Walsh 2013) and Tardigrada, although restricted to limno-terrestrial species (Bingemer et al. 2016; Sugiura et al. 2019; Bartel and Hohberg 2020). Representatives of other phyla, such as Loricifera (Kristensen 1983) or Gnathostomulida (Sterrer and Sørensen 2015), have never been observed while mating. Mating-associated behaviours in microscopic metazoans are a *tabula rasa*, despite the anatomy of reproductive system, and especially of parts presumed to be involved in copulation being an important character used in the systematics of some phyla, such as Gnathostomulida (Sterrer 1972) or Tardigrada (Degma and Guidetti 2018). Moreover, species identification in marine heterotardigrades also relies on genital anatomy (Kristensen and Hallas 1980; D'Addabbo Gallo et al. 1989; Kristensen and Higgins 1989; Pollock 1995; Bartels et al. 2018). Given that the genital traits have been extensively utilised in systematics, unravelling the ethological background of courtship and reproduction may likewise provide an insight into the classification of animals (e.g. Thiel and Duffy 2007).

In contrast to the exclusively microscopic tardigrades and loriciferans, the reproductive biology of phyla comprising both macrofauna and microfauna has been more thoroughly investigated and includes studies into the sexual dimorphism and parental care in annelids (Jouin 1968; Sella and Ramella 1999), mating behaviours in free-living planktonic nematodes and copepods (Jensen 1982; Uchima and Murano 1988; Buskey 1998), insemination in microscopic annelids and gastropods (Westheide 1978; Jörger et al. 2009), copulation in kalyptorhynchids and chaetognaths (Nagasawa 1985; Doe and Smith 2016) and parental care in amphipods and hermaphroditic polyclads (Thiel 1999; Rawlinson et al. 2008). The wide array of the reproductive biology topics unstudied, or poorly studied, in tardigrades means that we still lack even basic knowledge about reproduction in this group (Altiero et al. 2018).

Marine tardigrades belong predominantly to the class Heterotardigrada (Hansen 2011; Fontoura et al. 2017), with few representatives of the Eutardigrada inhabiting the sea (Ramazzotti 1972; Tsurusaki 1980; Kristensen 1982; Gąsiorek et al. 2019; Hansen and Kristensen 2020). Heterotardigrades have the gonopore separated from anus (Dewel et al. 1993; Møbjerg et al. 2018) and can be either gonochoristic (Jørgensen et al. 2014) or hermaphroditic (Suzuki and Kristensen 2014) taxa, the latter represented by only one species, *Orzeliscus belopus* du Bois Reymond Marcus, 1952 so far. Pacific florarctins (Halechiniscidae) have been the subject of systematic faunistic studies (Delamare-Deboutteville and Renaud-Mornant 1965; Renaud-Mornant 1967, 1987, 1989; Kristensen 1984; Noda 1987; Chang and Rho 1997; Jørgensen et al. 2014; Bartels et al. 2015; Fujimoto 2015). This subfamily is particularly interesting due to the

presence of epicuticular vesicles with endosymbiotic bacteria (Kristensen 1984), and a broad spectrum of sexual dimorphism, as sex-collection, is the goal of the present paper in *Ligiartus* Renaud-Mornant, 1982 (Gomes-Júnior et al. 2018) and *Wingstrandartus* Kristensen, 1984 (Kristensen 1984) but well marked in *Florarctus* Delamare-Deboutteville & Renaud-Mornant, 1965 (Renaud-Mornant 1989). Sex-specific differences have been described in recent papers about *Florarctus* from the Ionian Sea and the Caribbean (Hansen et al. 2016; Anguas-Escalante et al. 2020). In this paper, we elaborate on the sexual dimorphism and provide a detailed description of mating behaviour, in this mostly subtropical-tropical genus (e.g. Renaud-Mornant 1976; Gallo et al. 2007), observed in specimens collected from the Great Barrier Reef. This inclined us to summarise current knowledge regarding mating in tardigrades. As a corollary of our results, we conclude that descriptions of new arthrotardigrades belonging to genera with known sexually dimorphic species, and represented by only a single sex within samples, should be avoided as this may deepen the confusion in systematics caused by synonymies resulting from incomplete works.

Material and methods

In May of 1979, Niels Svennevig collected 12 samples of coralline sand from different coral reefs along the east coast of Australia and the Chesterfield Islands in the Coral Sea. The samples were collected from the surface of the sediments while diving at 2 m depth, and subsequently bulk fixed in 4% neutralised formaldehyde. Five years later, in the laboratory of the University of Copenhagen, tardigrades were extracted in fresh water by decanting the detritus from the samples through mermaid-bra nets with a mesh size of 30 µm and 62 µm. All the investigated samples contained arthrotardigrades, including specimens belonging to the genus *Wingstrandartus* (Kristensen 1984) and material from an atypical, undescribed species of *Florarctus*. The description of this last species, 40 years after its collection, is the goal of the present paper. Finally, new material of *Florarctus heimi* Delamare-Deboutteville & Renaud-Mornant, 1965 and *Florarctus cervinus* Renaud-Mornant, 1987 was collected from Shark Bay, Heron Island, between 1995 and 1996 (Kristensen 2003). Type material of these species was borrowed by Hansen (2011) from the National Museum of Natural History in Paris, where Jeanne Renaud-Mornant had held the position of curator of Tardigrada.

The detailed drawings of specimens on microslides were made based on specimens mounted in distilled water using a *camera lucida*. The water was subsequently replaced with glycerol, following an increased concentration series (5%, 10%, 25%, 50% and 100%), by placing the new media on

the side of the microscope slide coverslip. After the process, the animals were removed from the *camera lucida* slide, immersed in 100% glycerol or polyvinyl lactophenol (PVL; female NHMD-636745) and sealed using Glyceel®. The specimens on the microslides were studied using a light microscope with phase (PCM) or Nomarski (DIC) interference contrast. Micrographs were taken using an Olympus C-3030 zoom digital camera mounted either on an Olympus BX51 or on a Leica DM-RXA microscope. By drawing the specimens in distilled water, we were able to examine many structures that are otherwise invisible in glycerol-mounted material, and usually only reported from transmission electron microscopy (TEM) observations. These structures include different glands (“one-celled” glands, head glands, segmental ventral/dorsal glands and the two vesicles with symbiotic bacteria) and elements of the bucco-pharyngeal apparatus (stylets, placoids). The terminology follows Fontoura et al. (2017).

Results

Systematic account

Phylum: Tardigrada Doyère, 1840

Class: Heterotardigrada Marcus, 1927

Order: Arthrotardigrada Marcus, 1927*

Family: Halechiniscidae Thulin, 1928 (amended by Fujimoto et al. 2017)

Subfamily: Florarctinae Renaud-Mornant, 1982 (amended by Kristensen 1984)

Amended diagnosis: Halechiniscids with cuticular aliform expansions (*alae*) with or without *caestus*. Cephalic sense organs without tertiary clavae, strong secondary dimorphism in the primary clavae, secondary clavae transformed to dome-shaped papillae or H-shaped flat sacs. Usually, two to three cephalic vesicles containing symbiotic bacteria. Ventral segmental glands in all segments, opening near the insertion of coxae of the legs. Six to seven pairs of epidermal “one-cell” glands in total, found in the head and in the trunk. Four toes with claws present in adults, *digitus externus* with a hook-shaped *pedunculus*; *uncus* with *calcar externum*, *digitus internus* with a simple hook-shaped claw bearing a tiny dorsal spur. Males with ovoid gonopore close to anus. Females always with two seminal receptacles, each consisting of a spheroid vesicle with spermatozoa and an S-shaped genital duct opening laterally with a small papilla (anterior or posterior), entirely separated from the six-lobed (hexpartite/rosette) female gonopore. Anus three-lobed.

*Arthrotardigrada are a paraphyletic group with respect to the monophyletic Echiniscoidea (Fujimoto et al. 2017), and their systematic rank as an order is maintained herein only traditionally.

Genus: *Florarctus* Delamare-Deboutteville & Renaud-Mornant, 1965

Species: *Florarctus bellahelenae* sp. nov.

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Type material: Holotype (female) NHMD-636744, allotype (male) NHMD-636740, three paratypic males (NHMD-636741-3) and three paratypic females (NHMD-636745-7) that are in poor condition on the slides after 40 years. Table 1 provides the measurements for morphological characteristics. All type specimens and the remaining coralline sediment are preserved in the Natural History Museum of Denmark.

Type locality: North Cay of Long Island, Chesterfield Reefs (Pacific Ocean) at the GPS position: 19° 53' 00" S, 158° 19' 00" E.

Etymology: From Latin *bella* (masc. *bellus*) = beautiful + Helen; noun in genitive singular. The specific epithet has a twofold sense as (1) it honours Helene Møbjerg Boslev Kristensen for all the travels she has undertaken with us to collect tardigrades around the world; (2) Helen was the abducted queen of Sparta, sworn to prince Paris of Troy by Aphrodite. The morphology of *F. bellahelenae* strongly suggests that males, using their enormously long primary clavae, spend a substantial part of their life searching for (beautiful) females, as the Achaeans did in order to reclaim Helen, which ultimately resulted in the downfall of Troy.

Description of the holotypic female of *Florarctus bellahelenae* sp. nov. (Figs. 1 and 2)

NHMD-636744. Stored in a 100% glycerol microslide after fixation in 4% buffered formalin.

The body of the female is 149 µm long and 87 µm wide. Cuticle smooth, regularly (metamerically) folded on the dorsal side. The head (Figs. 1 and 2) is distinct from the trunk and has the following set of sense organs: cephalic cirri and clavae; the primary clavae (chemoreceptors) have a standard length (61 µm), and their shape is as in all other florarctin species. The median cirrus (mc, 19 µm) arises middorsally and is placed distally to the anterior margin of the head, which has a very large rostral *ala* (ra). Three gland cells are located at the base of the median cirrus (rg, Fig. 1), as in the allotype (see below). The internal cirri (31 µm) are placed dorsally, partly outside the rostral *ala* (ra). The external cirri (24 µm) are smaller and located ventrally. Cirrus A is difficult to measure, because it is covered by the primary clava, but its length is ca. 38 µm. The secondary clavae are fused (sc) and can be clearly observed from the ventral side in the holotype. The two vesicles with bacteria (vc) are very large. All four legs have setae (se₁ to se₄). The seta on the fourth leg has a blunt tip and is not spine-shaped. Cirrus E consists of a large cirrophore, a well-developed *scapus* with a characteristic spiral structure and a long *flagellum*. The *flagellum* comprises two parts: an internal layer, with a diameter almost the same as the *scapus*,

Table 1 Measurements (in μm) of selected morphological structures of the type series of *Florarctus bellahelenae* sp. nov.

Character	Holotype ♀	Allotype ♂	Paratypes				
			♂♂		♀♀		
			N	Range	N	Range	
Body length	149	85	3	101–110	3	123–144	
Body width	87	61	3	54–64	3	74–84	
Median cirrus	19	19	1	24	–	?	
Internal cirrus	31	31	2	37–44	–	?	
External cirrus	24	24	1	24	–	?	
Lateral cirrus A	38	28	2	32–33	–	?	
Primary clava	61	157	3	188–192	3	42–51	
Cirrus E	35	cf = 8 sp = 9 fl = 27	44	2	39–42	1	37
Sense organ on leg I	13	14	–	?	–	?	
Sense organ on leg II	12	12	–	?	–	?	
Sense organ on leg III	10	10	–	?	–	?	
Sense organ on leg IV	11	14	–	?	–	?	

N is the number of structures measured

and an external part, which is observable only under PCM. The entire cirrus is 35 μm long. As in many florarctin tardigrades, there are two particularly large lateral vesicles, containing symbiotic bacteria in all females (vc, Fig. 1). The two large unicellular structures (hg = head gland) and the three dorsal segmental glands (dg) cannot be observed in ventral view in the holotype. However, the ventral segmental glands (vg) are visible in the head and in all four trunk segments. In the trunk, the glands open close to the coxae. Only one pair of refractive glands, called “one-cell” epidermal glands (og, Figs. 1 and 5e), is present in the head of the holotype; however, five pairs exist in the trunk. The bucco-pharyngeal apparatus is more visible in the allotype.

The legs consist of coxa, femur, tibia and tarsus. The claws have unique structures found only in florarctins. A dorsal spur is present only on the internal claws, whereas each external claw has an *avicularium* appearing as a small notch, and a prominent dorsal *calcar*. Strong hook-shaped peduncles are seen at the base of the external digits in all leg pairs. The hooks of the peduncles are partly inserted in the tarsus.

The female reproductive system is fully mature. Only one large oocyte (ov) appears to mature at a time. Two seminal receptacles (rs) are filled with mature spermatozoa. The efferent genital ducts originating from these round vesicles are S-shaped and open, as two small papillae, posterolaterally from the six-lobed female gonopore. The female gonopore is located distally (20 μm) to the posteroventral trilobed anus.

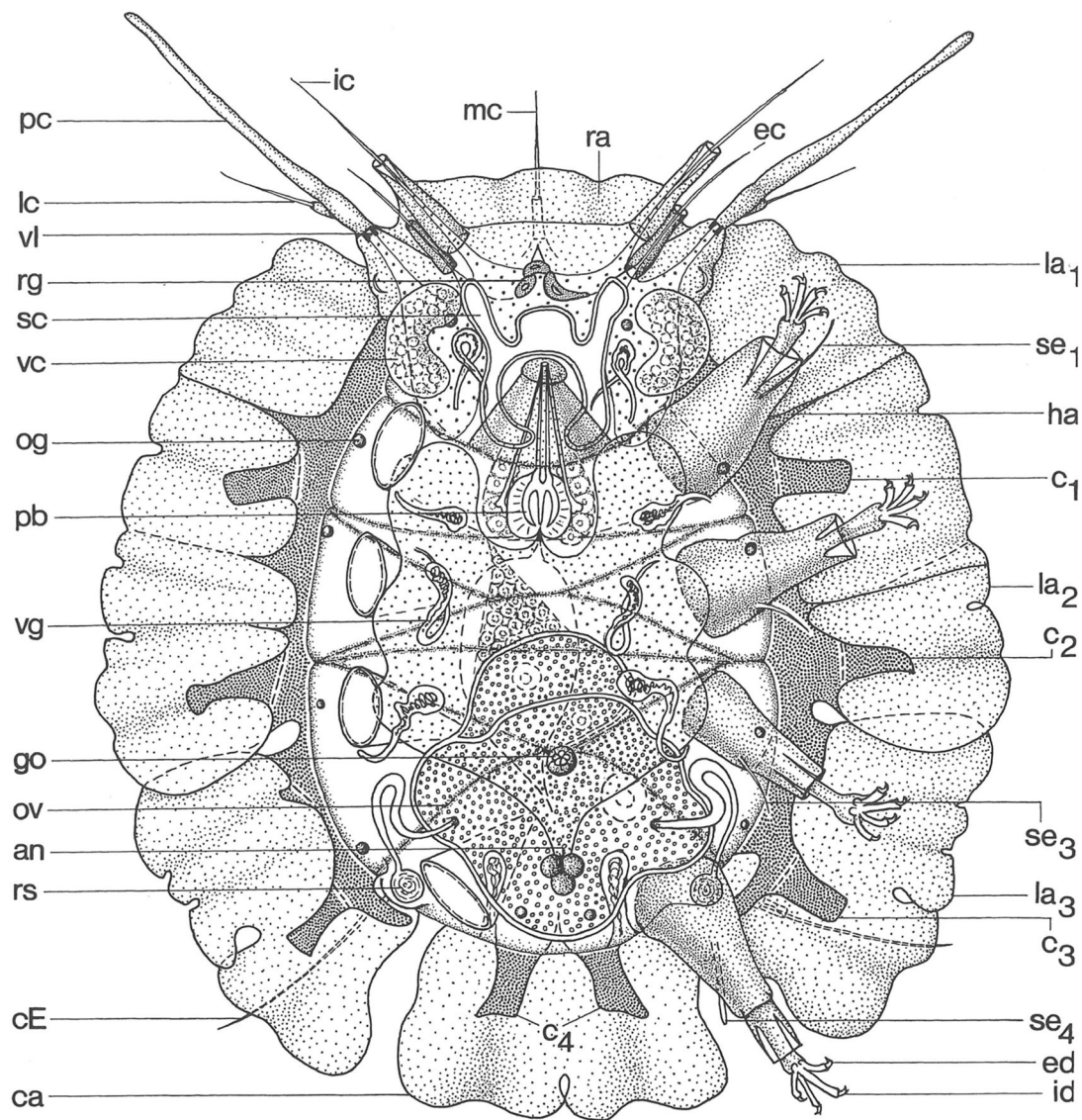
The *alae* consist of a very large rostral *ala* (ra) and two lateral *alae*, where the first and second lateral *alae* (la₁ and la₂) are clearly separated by a fold, and la₁ is further divided

into two unequal parts, hence the lateral *alae* are labelled as la₁ to la₃. The caudal *ala* is well developed and consists of four lobes. There are four pairs of *caesti* (c₁ to c₄): anterolateral c₁ at the level of legs I, and c₂ between legs II and III; posterolateral c₃ between legs III and IV; and simple (not ramified) caudal *caesti* c₄ (*caesti* do not extend laterally to the external edge of the *alae*; Fig. 1). These structures comprise both epicuticle with pillars, and procuticle (see Kristensen 1984). A new feature in *F. bellahelenae* is the long supporting structure in the first lateral *ala* (la₁) seen in both the holotypic female and the allotypic male. This new structure, the head *ala* support (ha), consists of the procuticle that extends to the external edge of the *ala*. The head *ala* support is located just in front of the first *caestus* (c₁). A similar structure appears to be present in the posterior part of the first lateral *ala* and forms the boundary between la₁ and la₂.

Description of the allotypic male of *Florarctus bellahelenae* sp. nov. (Figs. 3 and 4)

NHMD-636740. Data as for the holotype.

The body of the allotype is 85 μm long and 61 μm wide. The head and peribuccal cirri (Figs. 3, 4 and 5a) are identical to those in the holotypic female; however, the primary clavae are much thicker and nearly three times longer (157 μm) than those in the holotypic female (61 μm) and are thereby completely different from all other florarctin species or any other marine heterotardigrades. Furthermore, the allotypic primary clavae have an accordion-like outer structure (Fig. 5b), not seen previously in any arthrotardigrade. The median cirrus (mc, 19 μm) arises middorsally and is distant from the anterior margin of the head,



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Fig. 1 Habitus drawing of the holotypic female of *Florarctus bellahelenae* sp. nov. (ventral view). Locality: Long Island, Chesterfield Reefs (Pacific Ocean). List of abbreviations: an, anus; br, brain; c_{1-4} , caesti; ca, caudal ala; cE, cirrus E; cf, cirrophore; dg, dorsal segmental gland; ec, cirrus externus; ed, digitus externus; ex, ala with both internal (endocuticle) and external (epicuticle) supporting cuticle; fl, flagellum; go, gonopore; ha, long head supporting structure in the first lateral ala; hg, head gland; ic, cirrus internus; id, digitus internus; la_{1-3} , lateral alae;

lc, cirrus A; mc, cirrus medianus; og, “one-cell” epidermal gland; ov, oocyte in ovary; pb, pharynx; pc, primary clava; ra, rostral ala (= frontal ala in Fujimoto 2015); rg, rostral gland; rs, seminal receptacle (= receptaculum seminis); sc, secondary clava; se_{1-4} , sense organs on legs; sm, star-like dorsal muscle; sp, scapus; sv, seminal vesicle; sz, spermatozoa; te, testis; vc, cephalic vesicle with symbiotic bacteria; vg, ventral segmental gland; vl, van der Land’s body inside primary clava

which has a very large rostral ala (ra). At the base of the median cirrus, there are two to four rostral gland cells (more clearly seen in the holotypic female) (rg, Figs. 1 and 5d). The internal cirri (31 μ m) are placed dorsally, and part of the scapus (sp) and the entire flagellum (fl) extend beyond the rostral ala (ra). The external cirri are shorter (24 μ m) and located ventrally. The cirrus A length is about 28 μ m. The secondary clavae are fused but cannot be

observed from the dorsal side. The fourth leg seta (se_4) may be modified by having a blunt tip. Cirrus E is the same as in the female holotype (Fig. 5c). The whole structure is 44 μ m long. There are two small lateral vesicles containing symbiotic bacteria (vc, Fig. 3). Totally unique in the head of the allotype are two large unicellular structures (hg = head glands) similar to the dorsal segmental glands (dg) seen in the trunk. Although only three pairs

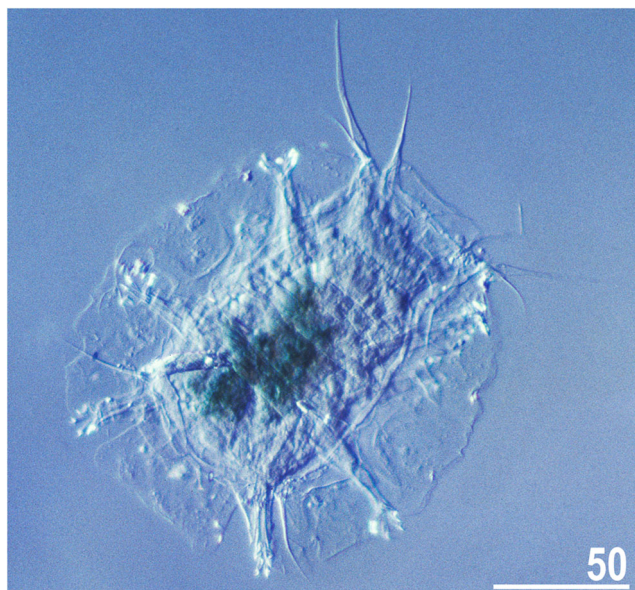


Fig. 2 Habitus image of the holotypic female of *Florarctus bellahelenae* sp. nov. (ventral view, DIC). Scale bar in μm

were observed, a fourth pair may be hidden under the large seminal vesicles (sv). A unique dorsal structure of the new species among all florarctin tardigrades is the star-like cross-striated muscle (sm) of the caudal segment (its presence in other taxa requires further investigation). The buccal tube is relatively short (ca. $19\ \mu\text{m}$ long). The stylets are short compared with other florarctins ($22\ \mu\text{m}$ long) and lack stylet supports (Fig. 5e, f). The pharyngeal bulb has three large apodemes in front of the three straight placoids. The placoids are short and slender. Unfortunately, all pharyngeal structures dissolved in the glycerol preparation.

The reproductive system comprises a small testis and two large seminal vesicles with two ventral ducts opening directly into the oval male gonopore located anteriorly of the trilobed anus. The gonopore/anus distance is very small ($2.5\ \mu\text{m}$). The seminal vesicles contain only about 100 late-stage spermatids with the characteristic wheat grain-shaped head. The tail (*flagellum*) in the spermatids is difficult to observe as the final stage of the male germ cells (spermatozoa) only develops in the female seminal receptacles.

Alae arranged as in the holotype. The first lateral *ala* is divided into two almost equal sections, with the anterior section the longest. *Caesti* inside the *alae* consist of single, unbranched projections (c_1 – c_4). The head *ala* support (ha) is present, but it is indistinct and does not appear to extend through the full width of the *ala*.

Differential diagnosis

The absence of any form of mammiliform sculpture on the dorsal cuticle (smooth with only metameric folds in *Florarctus bellahelenae* sp. nov.) and the presence of

continuous lateral *caesti* in the *alae* are shared by *Florarctus antillensis* van der Land, 1968, *Florarctus glareolus* Noda, 1987, *Florarctus pulcher* de Zio Grimaldi et al., 1999, *Florarctus wunai* Fujimoto, 2015 and *Florarctus yucatanensis* Anguas-Escalante et al., 2020 and the new species. Males of *Florarctus bellahelenae* sp. nov. can be easily distinguished from all described *Florarctus* species based on the accordion-like form of the primary clavae. Females can be distinguished from the following:

- *Florarctus antillensis*, based on the body size (*F. bellahelenae* sp. nov. does not reach $150\ \mu\text{m}$ in length vs up to $300\ \mu\text{m}$ in *F. antillensis*), and the sculpturing of leg setae (sculpture absent in *F. bellahelenae* sp. nov. vs the rugose cuticle present in *F. antillensis*);
- *Florarctus glareolus*, based on the position of the seminal receptacles (positioned far away from the gonopore, at the level of the anus in *F. bellahelenae* sp. nov. vs placed at the same distance from gonopore and anus in *F. glareolus*);
- *Florarctus pulcher*, based on the shape of the caudal *caesti* c_4 (pillar-like in *F. bellahelenae* sp. nov. vs club-shaped in *F. pulcher*);
- *Florarctus wunai*, based on the shape of the caudal *caesti* c_4 (pillar-like in *F. bellahelenae* sp. nov. vs papilla-shaped in *F. wunai*);
- *Florarctus yucatanensis*, based on the shape of both the lateral and caudal *caesti* (*caesti* simple, not lobed or ramified in *F. bellahelenae* sp. nov. vs anterolateral *caesti* with bilobed posterior projections, and caudal *caesti* with lateral projections in *F. yucatanensis*), and the dorsal cuticular wrinkling (absent in *F. bellahelenae* sp. nov. vs present in *F. yucatanensis*).

Species: *Florarctus heimi* Delamare-Deboutteville & Renaud-Mornant, 1965

Type material: Holotypic female (MNHN, Paris, A1278/50Ma) and one paratypic female (MNHN, Paris, MA8/551Ma). From New Caledonia.

New material: Additional specimens (Figs. 6, 7 8 and 9) from coralline sand collected by D. M. Kristensen and R. M. Kristensen at Shark Bay, Heron Island, Australia ($23^\circ 26.8' \text{ S}$, $151^\circ 55.18' \text{ E}$), on 13 December 1995, later deposited in the Zoological Museum, Copenhagen, Denmark (Kristensen 2003).

1. NHMD-672675. Two specimens: male and female mounted in glycerol. Male represents former *F. cervinus*. Male: length, $192\ \mu\text{m}$; width, $110\ \mu\text{m}$. Female: length, $285\ \mu\text{m}$; width, $163\ \mu\text{m}$.
2. NHMD-672676. Two specimens: male and female mounted in glycerol. Male represents former *F. cervinus*. Male: length, $170\ \mu\text{m}$; width, $94\ \mu\text{m}$. Female: length, $224\ \mu\text{m}$; width, $143\ \mu\text{m}$.

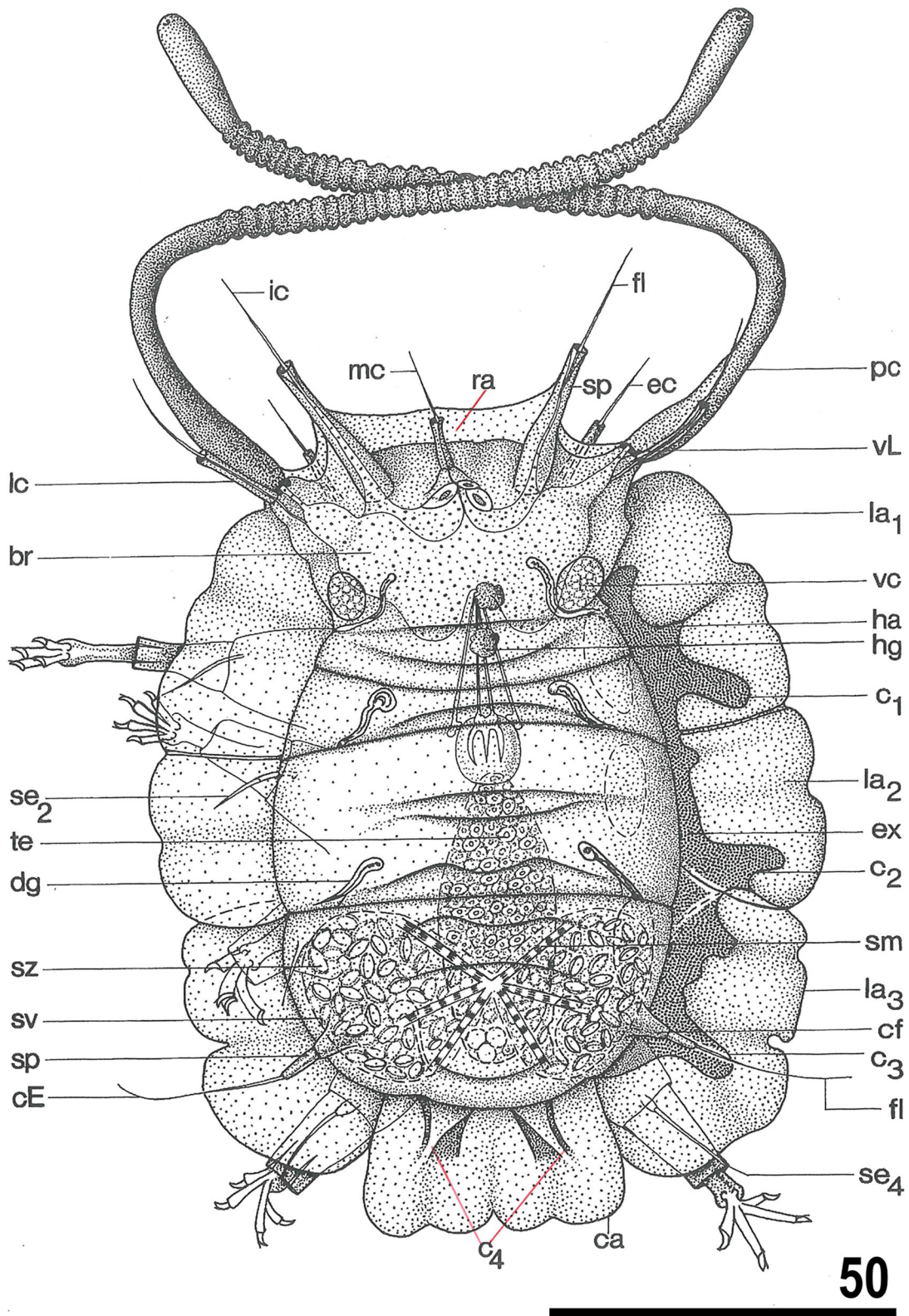


Fig. 3 Habitus drawing of the allotypic male of *Florarctus bellahelena* sp. nov. (dorsal view). Locality: Long Island, Chesterfield Reefs (Pacific Ocean). See the legend of Fig. 1 for abbreviations

- 3. NHMD-672678. Female mounted in glycerol. Length: 246 µm; width: 164 µm.
- 4. NHMD-672679. Male mounted in glycerol. Length: 176 µm; width: 101 µm.

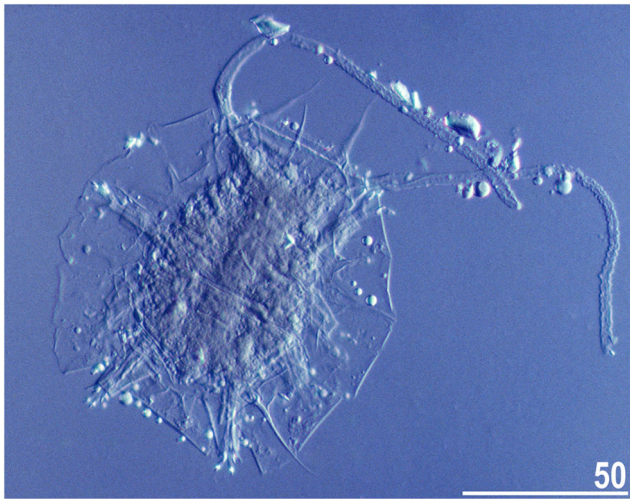
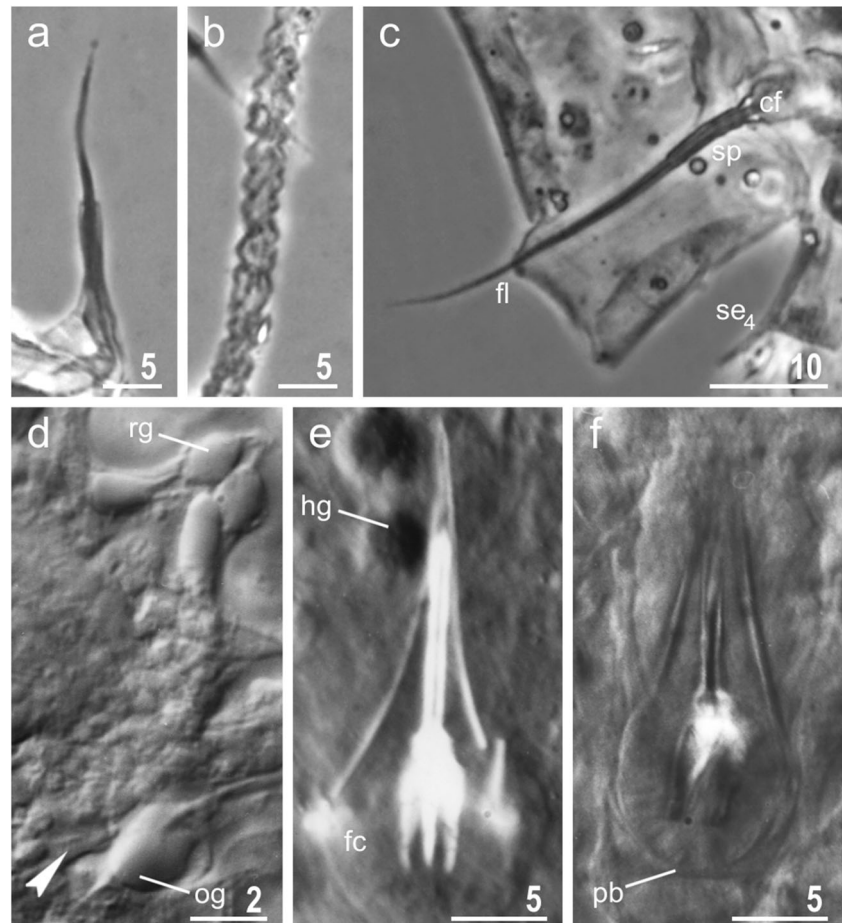


Fig. 4 Habitus image of the allotypic male of *Florarctus bellahelenae* sp. nov. (dorsal view, DIC). Scale bar in μm

While establishing the genus *Florarctus*, Delamare-Deboutteville and Renaud-Mornant (1965) made a detailed description of its type species *Florarctus heimi*. However, due to the lack of other comparable congeneric species (except

for *Florarctus salvati* Delamare-Deboutteville and Renaud-Mornant, 1965 described therein), some details were not studied attentively. The holotypic female is in the early stage of moulting. The new *alae* are captured during formation and lie folded beneath the old dorsal cuticle, along the lateral margin of the body. Neither new claws nor new sense organs are recognisable. The complete shape of the secondary clavae is evident as a large sense plate, almost completely encircling the mouth cone (Fig. 10a). The internal cirri are well developed, being proportionally much larger than in other congeneric species (Fig. 10b). Also, the fourth leg sense organ, which is usually an S-shaped papilla, is a long tapering spine (longer than cirrus *E*) with a basal swelling and a terminal pore (Fig. 10c). Each external claw has a strong *avicularium* and a large *calcar*, whereas each internal claw has a thin accessory hook and a long delicate *calcar*. Furthermore, each internal claw has a small but evident *avicularium* appearing as a small notch (Fig. 10d). The lateral *caesti*, which are poorly visible in the holotype, are more apparent in the paratype. Like in *F. antillensis*, the lateral *caesti* are continuous along the margin of the body, with only a few relatively small processes. The caudal *ala* is highly complex, although no *caestus* is evident. A deep median incision divides the *ala* into two large

Fig. 5 Microphotographs of *Florarctus bellahelenae* sp. nov. depicting morphological details (PCM). **a** cirrus internus; **b** surface of primary clava; **c** cirrus *E* and sense organ IV (**a–c** allotype); **d** internal head structures (female in distilled water; arrowhead indicates the frontal cephalic part); **e, f** buccopharyngeal apparatus (female and male in distilled water, respectively). List of abbreviations: cf, cirrophore; fc, furca; fl, *flagellum*; hg, head gland; og, “one-cell” epidermal gland; pb, pharynx; rg, rostral gland; sp, *scapus*. Scale bars in μm



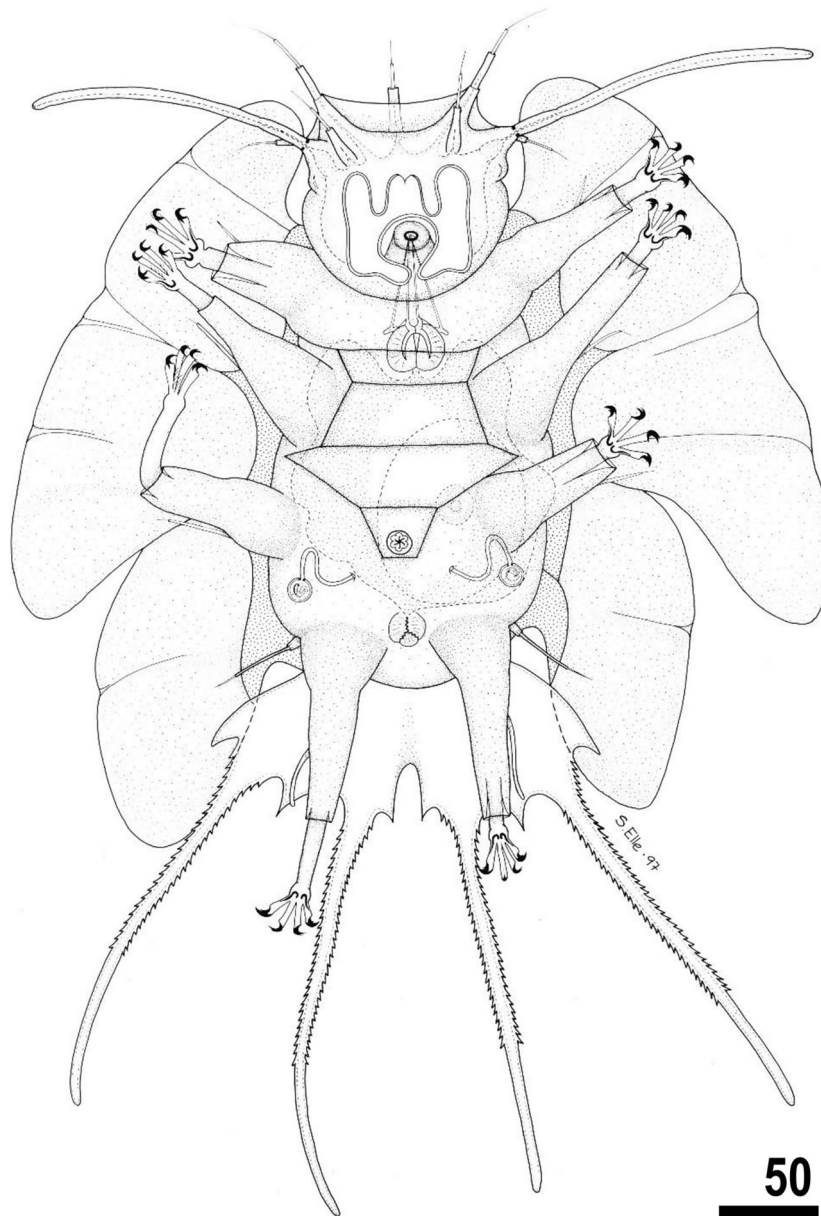


Fig. 6 Habitus drawing of the female of *Florarctus heimi* (ventral view). Locality: Shark Bay, Heron Island, Australia. Scale bar in μm

lobes. Each lobe terminates in two long and three short spines. The caudal *ala* spines are not evidently serrated in the holotype, whereas they are clearly serrated in the paratype. Each spine originates near the base of the *ala*: the short spines as internal string-like thickenings, and the long spines as broad thickenings (Fig. 10e). The paratype is in the late stage of moulting. As in the holotype, the new *alae* are formed and lie folded beneath the old dorsal cuticle, along the lateral margin of the body. Interestingly, the new caudal *ala* and spines are not folded. Instead, the new caudal *ala* has an orientation opposite to that of the old caudal *ala*, with the spines pointing anteriorly. New legs and claws are also evident. Examination of the Australian specimens collected from Heron Island allowed us to describe not only the bucco-pharyngeal

apparatus but also the male of *Florarctus heimi*. The stylets are thin and have large furcae; the placoids are large and curved. Three large and equally sized apophyses that appear as one highly refractive bulb are inserted anteriorly to the placoids (Figs. 6 and 7). Observations were also made of live individuals, some of which were found copulating. Surprisingly, the morphology of the males of *Florarctus heimi* cannot be distinguished from that of *Florarctus cervinus*. Based on this finding, we establish *Florarctus cervinus* as ***synonymum novum*** of *Florarctus heimi*. In conclusion, the descriptions of these two species, which were based on apparent morphological differences, in reality, represent the strong secondary sexual dimorphism displayed by *Florarctus heimi*. Consequently, the type material of *Florarctus cervinus*

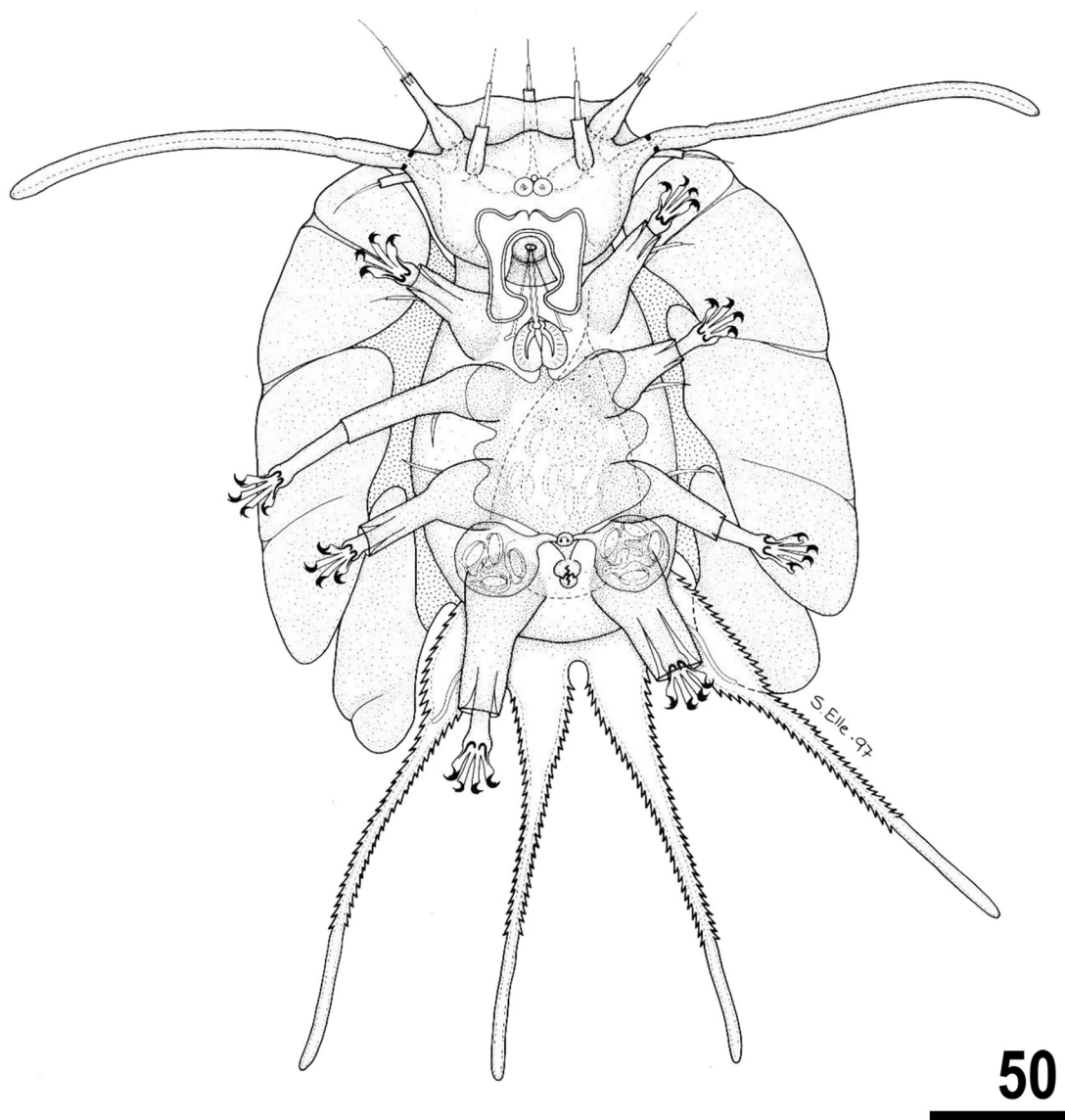


Fig. 7 Habitus drawing of the male of *Florarctus heimi* (ventral view). Locality: Shark Bay, Heron Island, Australia. Scale bar in μm

(holotypic male [MNHN, Paris, AR593] and two paratypes [MNHN, Paris, AR594, MA6/549Ma]) is included in the type material of *Florarctus heimi*, thus re-designating the holotype as the allotype. As we had the opportunity for the first time to compare males and females of *Florarctus heimi*, the most important sexually dimorphic differences are highlighted below. The anterior margin of the rostral (frontal) *ala* is approximately straight in the female, whereas in the male, it has a median indentation, forming two lobes. The configuration of the lateral *alae* is the same in both sexes; however, the posterior lateral *ala* is proportionally larger in the female (compare Figs. 6 and 8 with Figs. 7 and 9). The caudal *ala* represents the most conspicuous difference, with the female having four long spines and six short spines (Figs. 6, 8 and 10e) and the male having only four long spines (Figs. 7 and 9). Furthermore, the caudal *ala* of the female is larger and has a deep medial

indentation giving the impression there are two lobes. The caudal *ala* of the male has less deep and broadly ovate median indentation compared to that of the female (compare Figs. 6 and 7).

Differential diagnosis *Florarctus heimi* is currently the only representative of the genus characterised by caudal *ala* with long serrated spines.

Discussion

Anatomy

Several structures new to tardigrade anatomy are introduced in this contribution. The ventral segmental glands

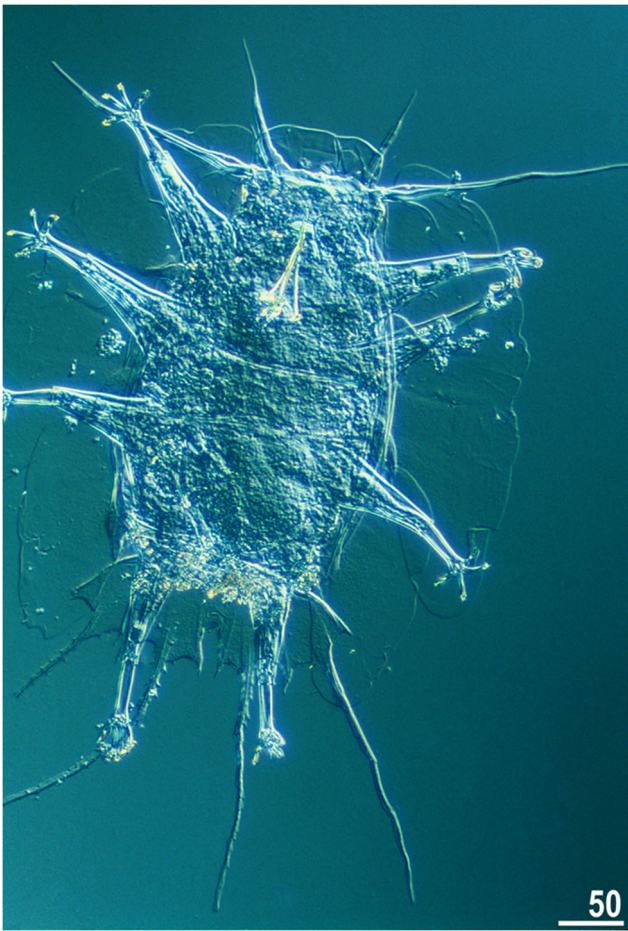


Fig. 8 Habitus image of the female of *Florarctus heimi* (ventral view, DIC). Scale bar in μm

were first observed in *Wingstrandarctus* (Kristensen 1984) and subsequently found in nearly all arthrotardigrades. Given their location inside the organism, and orifices that open near the legs, these glands are considered to be excretory organs similar to the coxal glands in marine xiphosurans (Briggs and Moss 1997). The ‘one-cell’ epidermal glands undergo structural modifications during the moulting cycle and may therefore be involved in the formation of the new cuticle. These structures were first described in all species of *Wingstrandarctus*, but Renaud-Mornant (1989) subsequently reported their presence in *Florarctus*. Two to four gland cells (rostral glands) are located at the base of the median cirrus (Figs. 1 and 5d), and their putative function remains to be investigated. Head glands are present in males and identifiable as large unicellular structures. Their presence in other florarctins is yet to be demonstrated. In common with many florarctin tardigrades, including *Ligiartus* (Gomes-Júnior et al. 2018), there are only two lateral vesicles with symbiotic bacteria (vc, Fig. 3) in *Florarctus bellahelenae* sp. nov., and not three as in *Wingstrandarctus* (Kristensen 1984). As *Ligiartus* was

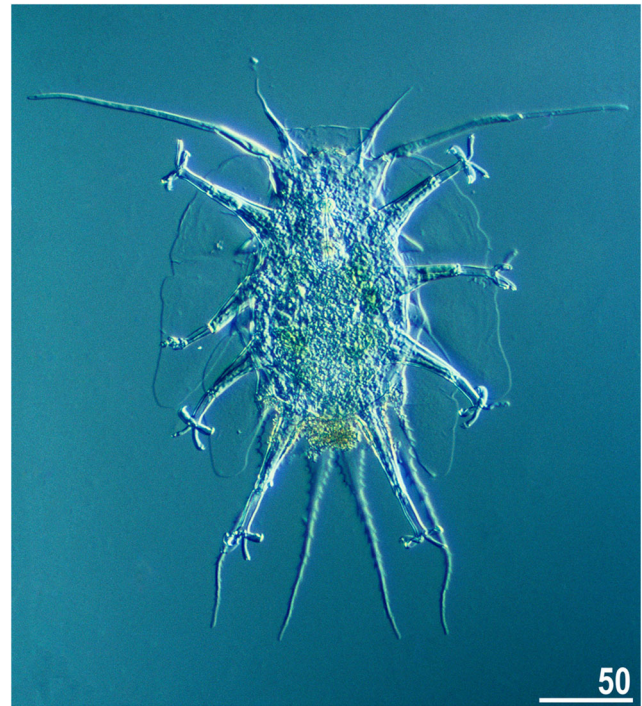


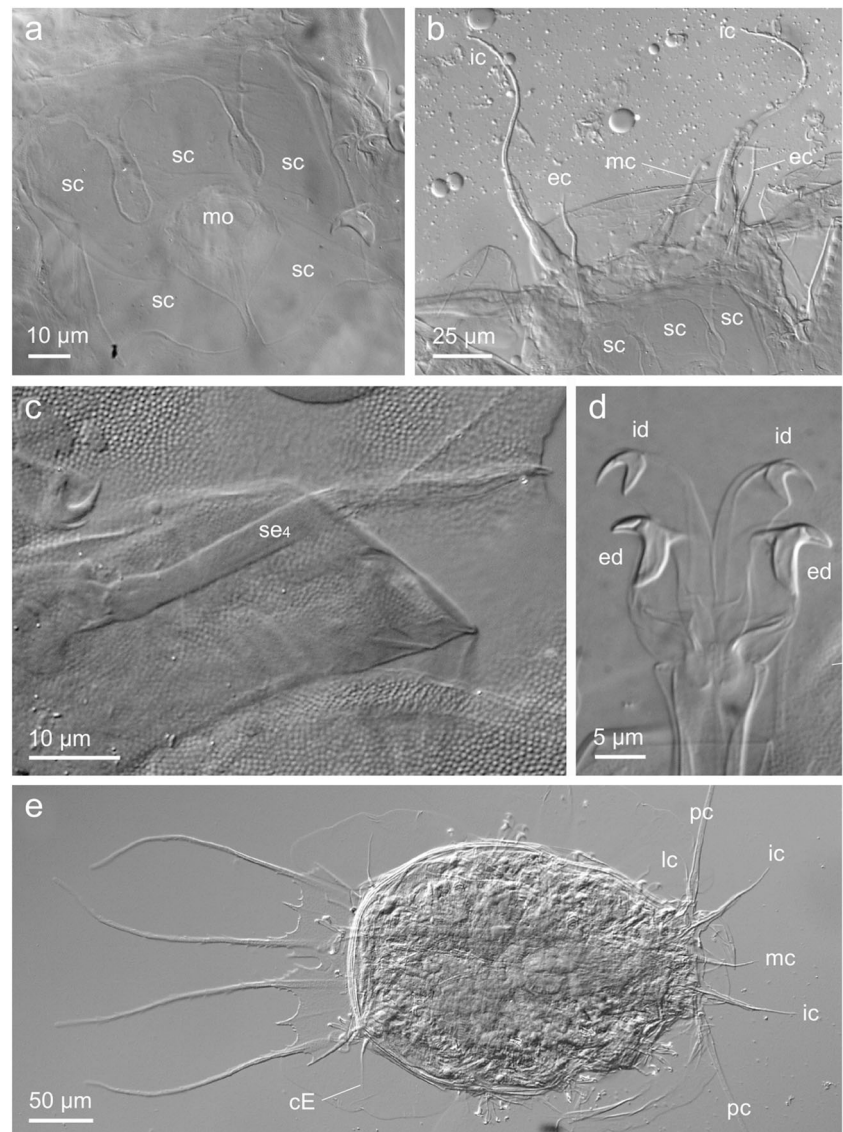
Fig. 9 Habitus image of the male of *Florarctus heimi* (ventral view, DIC). Scale bar in μm

inferred to be the sister group of the remaining florarctins (Hansen 2011), it is hypothesised that the additional vesicle is an apomorphy of *Wingstrandarctus*.

Mating behaviours and copulation

A summary of knowledge relating to tardigrade pre-mating, mating and copulatory behaviours is presented in Table 2. Strong sexual dimorphism in eutardigrades is rare and restricted to modifications of the claws on the first leg pair in males, an adaption for holding females during mating (Rebecchi and Nelson 1998; Gašiorek et al. 2019). Recently, detailed descriptions of mating positions and sexual reproduction behaviours have been provided for representatives of Isohypsibiidae and Macrobiotidae. These observations, together with the first notes on the enticing actions males perform to initiate courtship in *Batillipes noerrevangi* Kristensen, 1978 (Kristensen 1979), indicate an active role for males. The probability of internal fertilisation was hypothesised for macrobiotids based on the position of the spermatheca with respect to the rectum (Sugiura et al. 2019). Elsewhere, the reproductive system of all marine tardigrades had been thoroughly investigated (Pollock 1970; Renaud-Mornant 1982). However, there was a problem with the interpretation of the so-called ‘annex glands’ in the family Stygarctidae and the lateral vesicles in the subfamily Florarctinae. Transmission electron microscopy investigations clearly showed that both the ‘annex glands’ and the lateral vesicles are seminal receptacles (Kristensen 1984); thus, all arthrotardigrades likely exhibit internal

Fig. 10 Microphotographs of the holotype of *Florarctus heimi* depicting morphological details (DIC). **a** secondary clavae surrounding the mouth opening; **b** peribuccal cirri surrounding the mouth cone; **c** sense organ of leg IV; **d** claws (ed, external digit; id, internal digit); **e** habitus in dorsal view, showing the spine structure inside the caudal *ala*



fertilisation (it needs to be stressed that seminal receptacles have not been detected in batillipedids — only enigmatic spermathecae were reported by Grimaldi de Zio and D'Addabbo Gallo 1975). Importantly, the two seminal

receptacles are surrounded with cuticle so oocytes can be fertilised when a female lays freshly formed eggs into it: one in *Actinarctus* (Jørgensen et al. 1999) or *Florarctus* (Hansen et al. 2016) and up to eight eggs in *Tetrakentron* (Kristensen

Table 2 Summary of observations on reproductive behaviours of tardigrades

Species	Class	Family	Main findings	Reference
<i>Batillipes noerrevangi</i>	Heterotardigrada	Batillipedidae	Initiation of courtship reported	Kristensen (1979)
<i>Florarctus bellahelenae</i> sp. nov.	Heterotardigrada	Halechiniscidae	Observation of ventral mating	This study
<i>Isohypsibius dastychi</i>	Eutardigrada	Isohypsibiidae	The first comprehensive description of courtship	Bingemer et al. (2016)
			Indirect evidence for pheromone signalling	Bartel and Hohberg (2020)
<i>Macrobotus shonaicus</i>	Eutardigrada	Macrobotidae	The second comprehensive description of courtship	Sugiura et al. (2019)
<i>Milnesium inceptum</i>	Eutardigrada	Milnesiidae	Indirect evidence for sexual signalling	Suzuki (2008)
<i>Parastygarctus sterreri</i>	Heterotardigrada	Stygarctidae	Observation of ventral mating	This study
<i>Pseudobiotus megalonyx</i>	Eutardigrada	Doryphoribiidae	Ejaculation of spermatozoa into exuvium reported	von Erlanger (1895)

1980). Until now, there has only been one observation of a heterotardigrade mating behaviour in a member of Stygarctidae, *Parastygarctus sterreri* Renaud-Mornant, 1970, from Bermuda (R.M. Kristensen, pers. obs.). Mating ending with internal fertilisation, both in a stygarctid and in a florarctin, has been referred to as “venter-to-venter”.

Taxonomy

Males are typically smaller than females in florarctins. The fact that males of *F. heimi* and females of *F. cervinus* were previously unknown, combined with the fact that both species were described from New Caledonia, should have raised some doubts regarding potential conspecificity. In December 1995, during the Australian summer, large populations of a *Florarctus* species were found in coralline sand collected subtidally in Shark Bay, Heron Island (the southern Great Barrier Reef). Analysis revealed two species were present, *Florarctus heimi* and *F. cervinus*, and that they were always found together. In vivo observations revealed that all specimens of the larger *F. heimi* (up to about 400 µm) were females. In contrast, all adults of the smaller *F. cervinus* (about 170 µm) were males. The differences in the caudal expansions of *alae* between the two putative species were congruent with the original descriptions. Moreover, males of *F. cervinus* were observed mating with females of *F. heimi*.

In conclusion, the morphological and behavioural evidence indicates that *F. cervinus* is a junior synonym of *F. heimi*. It is known for other microscopic animals that describing species based on individuals of a single sex might lead to an inflation of the number of species (e.g. Karanovic 2008). Only molecular data could most objectively test the assertion of their conspecificity, analogously to actions undertaken by crustacean specialists in the copepod order Monstrilloida (Jeon et al. 2018) to link sexes of one species. As a consequence, new marine heterotardigrades with strongly sexually dimorphic traits should not be described based on individuals of one sex, if only to avoid future synonymies.

Biogeography

The vast majority of records of *Florarctus* fall within two regions: warm oceans and warm-temperate provinces (Briggs and Bowen 2012). There are only two reports of *Florarctus* species (*Florarctus acer* Renaud-Mornant, 1989 and *Florarctus hulingsi* Renaud-Mornant, 1976) from cold waters of the Bay of Morlaix, North Atlantic Ocean (Renaud-Mornant and Gourbault 1980; Renaud-Mornant 1989). All records of *Florarctus* published since the last summary of marine tardigrade zoogeography (Kaczmarek et al. 2015) originate from tropical or subtropical areas (Fujimoto 2015; Bartels et al. 2018; Anguas-Escalante et al. 2020); therefore, we hypothesise that such clear temperature-driven

regionalisation of an entire genus of marine tardigrades is uncommon; however, it has never been rigorously tested. If positively verified, such regionalisation would appear unusual as the species composition of marine tardigrade communities changes with depth (Hansen et al. 2001), exhibiting bathymetric-dependent diversity and endemism patterns known for other animals (Rex 1973, 1981).

There are five *Florarctus* species: *F. antillensis*, *F. asper* Renaud-Mornant, 1989, *F. cinctus* Renaud-Mornant, 1976, *F. hulingsi* and *F. stellatus* Renaud-Mornant, 1989, that are characterised by very wide distributions, always embracing many records from the relatively well-sampled Mediterranean region and usually only some tropical locales (Kaczmarek et al. 2015). This situation seems incongruous given the recently revealed species complex in the genus *Batillipes* (Santos et al. 2019) from the Atlantic Ocean and the Mediterranean. Consequently, geographic ranges should be better documented in tardigrades if claims, for example that species occurring in both the Mediterranean and the Coral Sea are the same, are to be supported. In the light of this, a single report of *F. heimi* from the Maldives (Gallo et al. 2007) raises concerns about the validity of that identification, particularly as it was not supported by a more elaborate description. The presence of *F. heimi* in the Indian Ocean should therefore be treated with caution. A lack of taxonomic knowledge relating to the delineation of morphologically similar species similarly contributed to misleadingly wide geographic ranges in some marine animals (e.g. Cerca et al. 2020). Conversely, evidence from other meiofaunal groups supports a cosmopolitan distribution in many cases (e.g. Meyer-Wachsmuth et al. 2014).

Supplementary Information Live male of *Florarctus heimi* collected in Shark Bay, Heron Island; Australia. The male just after mating with a female. One of the primary clavae is broken after the copulation. The online version contains supplementary material available at <https://doi.org/10.1007/s12526-021-01183-y>.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for animal testing, animal care and use of animals were followed by the authors.

Sampling and field studies All necessary permits for sampling and observational field studies were obtained by the authors from the competent authorities and are mentioned in the Acknowledgements. The study is compliant with CBD and Nagoya protocols.

Data availability All data generated or analysed during this study are included in this published article and its supplementary information files.

Author contribution RMK conceived and designed the research. DMK and RMK conducted the sampling and field observations. PG and RMK analysed the data. All authors wrote, read and approved the manuscript.

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