Do eutardigrades have a copulatory organ? The case of *Halobiotus crispae* Kristensen, 1982 (Tardigrada: Halobiotidae)

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ABSTRACT: Reproductive strategies of tardigrades vary considerably among taxa. Species of Heterotardigrada have separate gonopore and anus, whereas Eutardigrada species have a single cloacal opening, which is a shared exit from the cloaca, hindgut, and Malpighian tubules. While internal fertilization has been hypothesized for certain species based on the presence of spermatozoa in female spermathecae, the mechanism of sperm transfer remains undocumented. Notably, sexual dimorphism has not previously been reported in eutardigrades. In this study, males and females of the eutardigrade *Halobiotus crispae* Kristensen, 1982 were collected from sand samples in the intertidal zone of Olenevsky Island, White Sea, and analyzed using molecular, electron-microscopy and immunocytochemical methods. Our findings advance the understanding of *H. crispae* reproductive biology, including the first documented evidence of sexual dimorphism in eutardigrades. Males possess a protrusible cloacal cylinder, hypothesized to function as a copulatory organ. Distinct morphological differences in cloacal structure were observed in two sexes. Males have oval-shaped and smaller cloaca with smaller cloacal plates; females have larger, diamond-shaped cloaca, with acute-triangular cloacal plates. Additionally, sensory organs and musculature near the cloacal plates and in the fourth pair of legs have been found which suggest neural regulation during mating, potentially guiding copulatory interactions. The proposed role of the male cloacal cylinder as a copulatory organ supports the hypothesis of internal fertilization in this group, changing the current understanding of reproductive strategies in tardigrades. These findings underscore the need for deeper exploration of tardigrade reproduction, particularly to clarify the prevalence and mechanisms of internal fertilization across species.
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KEY WORDS: Tardigrada, sexual dimorphism, reproduction, fertilization, molecular genetics, scanning electron microscopy, immunocytochemistry.

Devoted to memory of Claus Nielsen.

Обладают ли эутардиграды копулятивным органом? Случай *Halobiotus crispae* Kristensen, 1982 (Tardigrada: Halobiotidae)

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РЕЗЮМЕ: Репродуктивные стратегии тихоходок значительно различаются между таксонами. Виды Heterotardigrada имеют отдельные гонопор и анус, а виды Eutardigrada имеют клоакальное отверстие, которое является общим выходом из клоаки, задней кишки и Мальпигиевых сосудов. Для нескольких видов, у которых сперматозоиды были обнаружены в семяприемниках самок, предполагается внутреннее оплодотворение, но способ передачи спермы до сих пор неизвестен. Половой диморфизм ранее не был отмечен у эутардиград.
Самцы и самки эутардиграды *Halobiotus crispae* Kristensen, 1982 были обнаружены в образцах песчаного грунта в приливно-отливной зоне острова Оленевский Белого моря, и изучены молекулярными, электронно-микроскопическими и иммуноцитохимическими методами. Самцы обладают выдвижным клоакальным цилиндром, предположительно функционирующим как копулятивный орган. Были обнаружены четкие различия в морфологии клоаки у самок и самцов. У самцов клоака овальная, меньшего размера; у самок клоака крупнее, ромбовидной формы, с остро-треугольными клоакальными пластинами. Это первый задокументированный случай внешнего полового диморфизма у эутардиград. Были обнаружены мускулатура и сенсорные органы клоакальных пластин четвертой пары ног, что предполагает нервную регуляцию в процессе спаривания и копулятивных взаимодействий. Роль мужского клоакального цилиндра как копулятивного органа предполагает внутреннее оплодотворение у эутардиград, что меняет имеющиеся представления о репродуктивных стратегиях этой группы тихоходок. Результаты подчеркивают необходимость более глубокого изучения размножения тихоходок, в частности, для выяснения распространенности и механизмов внутреннего оплодотворения у разных видов.
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КЛЮЧЕВЫЕ СЛОВА: Tardigrada, половой диморфизм, размножение, оплодотворение, молекулярная генетика, сканирующая электронная микроскопия, иммуноцитохимия.

Introduction

Halobiotus crispae Kristensen, 1982 is a secondarily marine tardigrade belonging to the class Eutardigrada, order Parachela, family Halobiotidae (Gąsiorek *et al*., 2019; Degma *et al*., 2024). The species was described in 1982 by Reinhardt Kristensen, a Danish zoologist, and is the type species of the genus *Halobiotus* (Kristensen, 1982). It inhabits the intertidal and subtidal zones within algae thickets (Møbjerg *et al*., 2007).

Tardigrades are known to exhibit either gonochorism (with separate male and female individuals) or hermaphroditism. Reproduction occurs exclusively through gametes (Bertolani, 1983, 1990, 2001). Reproductive strategies of tardigrades include amphimixis (cross-fertilization or self-fertilization) and thelytokous parthenogenesis (apomixis or automixis) (Bertolani, Rebecchi, 1999; Bertolani, 2001; Nelson *et al.*, 2015; Altiero *et al.*, 2018). In tardigrade populations, sex ratios vary greatly (Rebecchi, Bertolani, 1994). Males are most often few, extremely rare, or completely unknown (Ramazzotti, Maucci, 1983; Suzuki, 2008; Gąsiorek *et al*., 2020). There may also be seasonal differences in the presence of males in populations (Gąsiorek *et al*., 2020). Internal fertilization has only been supposed when a spermatheca full of non-motile spermatozoa was found within the female (Bertolani, Rebecchi, 1999; Stec *et al.*, 2021; Vecchi *et al.*, 2022). However, in other cases, internal fertilization has not yet been reliably documented (Bertolani, 1983, 1990, 2001; Bertolani, Rebecchi, 1999; Sugiura, Matsumoto, 2021a,b). The reproductive system of tardigrades consists of an unpaired gonad (ovary or testis, or hermaphroditic gonad – ovotestis) and genital ducts – two in males and one in females and hermaphrodites. Seminal vesicles in males and spermathecae in females have been described in some species (Bertolani, Rebecchi, 1999). Species of Heterotardigrada have separate gonopore and anus, but Eutardigrada species lack external gonopores as they have a cloaca where the hindgut, Malpighian tubules and gonoducts terminate (Nelson *et al.*, 2015). In some marine and terrestrial heterotardigrades, male gonopore opens at the top of a small, externally protruding tube that could facilitate copulation (Bertolani, 1990). Ventral cloaca of eutardigrades is similar in both sexes (Bertolani, Rebecchi, 1999). External sexual dimorphism or copulatory organs have never been observed in eutardigrades.

Halobiotus crispae is characterized by cyclomorphosis (Kristensen, 1982). The phenomenon includes 4 main stages: 1) pseudosimplex 1 (winter form): the hibernation stage, characterized by the absence of mature gonads, a double cuticle and continuous placoids in the form of stripes, long claws, the mouth and cloaca are closed with cuticular thickenings; 2) pseudosimplex 2 (spring form): a sexually maturing stage occurring in spring after shedding the old cuticle. This stage is characterized by developing gonads, relatively small claws, fused placoids in stripes, and a small open mouth; 3) active stage (summer form): gonads and claws completely developed, the mouth and cloaca are open, and six peribuccal sensory organs are present; the pharyngeal placoids are divided into macro- and microplacoids; 4) simplex (summer form): a molting stage, also characteristic of sexually mature animals; it is initiated by shedding of the buccopharyngeal apparatus, which is newly developed during different stages of the simplex, the mouth and cloaca are closed, and peribuccal sensory organs are absent (Kristensen, 1982, Møbjerg *et al*., 2007; Halberg *et al*., 2009b).

The females of *Halobiotus* lay eggs in the exuviae during moulting (Kristensen, 1982). Usually, eggs in the exuviae have smooth shell (Altiero *et al*., 2018), formed inside the female. An oocyte and a gonad wall participate in this process (Poprawa, 2011). It was suggested that *H. crispae* was characterized by external fertilization (Kristensen, 1982) since sperm morphology is of a "primitive" type (normal acrosome, helicoid nucleus and untransformed mitochondria). Also, no spermathecae or other signs of internal fertilization were found (Kristensen, 1982). To date, there are no additional data in the literature about fertilization process in this species.

Here we present the first observation of sexual dimorphism in cloacal structure and provide the first description of a cloacal cylinder in males of *Halobiotus crispae.* Based on its morphology, this structure may function as a copulatory organ.

Material and methods

Sampling

Sandy sediment samples were collected in June 2022 in the intertidal zone of Olenevsky Island (Kandalaksha Bay of the White Sea) 66°31′19.6″N,

33°07′31.4″E. The samples contained numerous tardigrades. Animals were sorted under a stereomicroscope using a capillary pipette and placed in fresh water for 1–2 min for relaxation. A total of 18 samples were investigated. Several individuals exhibiting a tubular protrusion at the caudal end between the 4th pair of legs were selected for scanning electron microscopy examination.

Light microscopy (LM)

Specimens were fixed in 4% formaldehyde in distilled water neutralized with $CaCO_3$, washed in distilled water and stored in Seinhorst liquid (0.5% glycerin + 30% alcohol). The specimens were examined using a Leica CTR5000 light microscope.

Scanning electron microscopy (SEM)

For scanning electron microscopy, specimens were fixed in 2.5% glutaraldehyde on 0.1 M sodium phosphate buffer (PBS) and posfixed in 1% OsO_4 on PBS, pH 7.4, dehydrated in increasing concentrations of ethanol, then in a mixture of 96% ethanol and 100% acetone in an increasing gradient for 10 min, and in 100% acetone twice for 10 min. The samples were critical point dried using liquid CO_2 (HCP-2 Critical Point Dryer, Hitachi, 1980), sputter coated with a platinum-palladium mixture (EIKO IB-3 Ion Coater, 1980) and examined using a Thermo Fisher Scientific Quattro S SEM. 6 specimens were examined using SEM.

Confocal laser scanning microscopy (CLSM)

For immunocytochemical examination in a CLSM, the samples were fixed within 2 hours in 4% paraform on PBS, pH 7.4, exposed to ultrasound for 30 and 50 sec ultrasound power 120 W, frequency 40 kHz, voltage AC220 - 240 W, washed in PBS twice for 10 min and kept in a solution of 7% Triton X100 in 0.1 M PBS with the addition of 0.03% NaN3 on a shaker at 4 °C for two days. Then samples were incubated in a solution of antibodies against α-tubulin. As primary antibodies, we used mouse monoclonal anti-acetylated-tubulin (SIGMA, USA), clone 6 –11 B -1, product number: T6793 in a concentration of 1:1000 diluted in 0.1 M PBS with the addition of 1% Bovine Serum Albumin (BSA), 1% Dimethyl sulfoxide and 1% Triton X100 (TBP solution) for 3 days. After several washes in TBP solution (8 times for 30 min), the samples were incubated in a solution containing the secondary antibody: anti-mouse AlexaFluor 488 from goat in a concentration of 1:1000 diluted in 0.1 M PBS with 1% DMSO for one day and then were washed 2 times for 30 min in the same buffer. After that the samples were stained with nuclear dye DAPI in 0.1 M PBS, at a concentration of 1:500 and washed with 0.1M PBS 10 times for 20 min. All procedures were carried out on a shaker at 4 °C. The samples were put in gradient glycerol series with the addition of 2.5% DABCO (1,4-diazabicyclo[2.2.2] octane): 30% glycerol overnight, 60% 1,5 hour and 70% 1,5 hour. The preparations were examined in a Nikon A1 confocal microscope at wavelengths 405 nm and 488 nm. Two individuals were examined. It should be noted that the cuticle and tissues of tardigrades have high autofluorescence in wide range.

DNA extraction, PCR, and sequencing

Four fixed in 96° ethanol individuals of *Halobiotus crispae* were treated with proteinase K at 50°C for 24 hours. After that total genomic DNA was extracted using the DIAtom DNA Prep Kit (Isogen, Russia) following the protocol provided by the manufacturer. Two regions – nuclear ribosomal (containing partial 18S and 28S rRNA genes, complete 5.8S rRNA gene, and ITS1 and ITS2 sequences) and mitochondrial (partial gene *cox1*) – were amplified using primers (5′-GTATCTGGTTGATCCTGCCAGT-3′ and 5′-ATATGCTTAARTTCAGCGGGT-3′ for nuclear rDNA and 5’-GGTCAACAAATCATAAAGATATTGG-3’ and 5’-TAAACTTCAGGGTGACCAAAAAATCA-3’ for *cox1*) with Encyclo PCR kit (Evrogen, Russia). The PCR cycling conditions for rDNA sequence included denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 3 min, and final extension at 72 °C for 5 min. The PCR cycling conditions for *cox1* sequence included denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 43 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. PCR products were separated with agarose gel electrophoresis and purified using Cleanup Mini kit (Evrogen, Russia). Amplicons were sequenced directly with an Applied Biosystems 3730 DNA Analyzer. The sequences of *H. crispae* were deposited in GenBank with accession numbers PP537167 for nuclear rDNA (3787 bp) and PP535567 for *cox1* gene fragment (686 bp).

Sequence alignment

Obtained nuclear rDNA was aligned with three sequence of *H. crispae* from GenBank database (Accession Numbers EF620420, EF620421, and EF620422; Møbjerg *et al*., 2007) using MAFFT online service (Katoh *et al*., 2019) for accurate prediction of ITS2 region localization. The secondary structure of nuclear ITS2 regions were predicted using RNAstructure software (Reuter, Mathews, 2010) with default values. All predicted structures of ITS2 were manually examined and compared for common stems, loops, and bulges. Compensatory base changes (CBCs) in different helices were manually identified for all ITS2 secondary structures.

Results

MOLECULAR ANALYSIS. 18S rRNA and cox1 gene sequences of the White Sea *H. crispae*

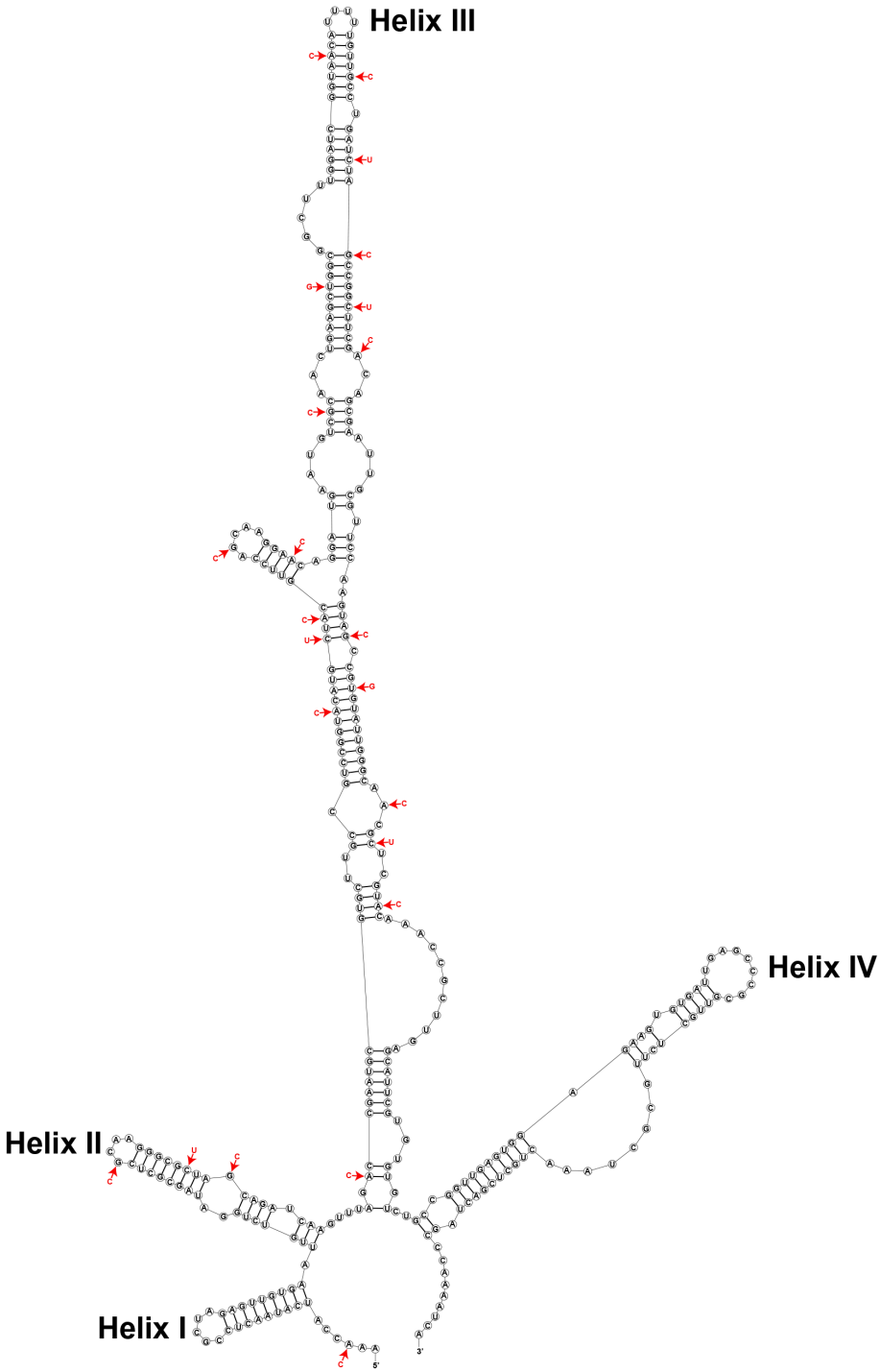

Fig. 1. Predicted secondary structure of ITS2 for *Halobiotus crispae* based on the nucleotide sequence of *H. crispae* from Baffin Bay, the North and Baltic Seas, red arrows indicate nucleotide substitutions found in the White Sea specimens. Helices are numbered according to Coleman (2000).

are 100% identical with *H. crispae* isolates from GenBank database. ITS2 sequence of the White Sea *H. crispae* is 93% identical with the three identical to each other *H. crispae* isolates from different locations (Baffin Bay, North Sea, and Baltic Sea) (GenBank EF620420-2; Møbjerg *et al*., 2007). The predicted ITS2 secondary structure of *H. crispae* folded into a typical structure with four common major helices as in most eukaryotes (Coleman, 2000, 2007), with the longest helix III (Fig. 1). The White Sea isolate differs from the other isolates by 23 substitutions, however, there is not a single compensatory base change (CBC) substitution. The absence of CBCs between *Halobiotus* isolates indicates the putative absence of genetic isolation between these isolates which in turn confirms their conspecificity (Coleman, 2000, 2007; Wolf *et al*., 2013). Thus, the White Sea *Halobiotus* isolate identification to *H. crispae* was confirmed with molecular data.

EXTERNAL MORPHOLOGY. Specimens of *H. crispae* included adults at the active and simplex stages. Individuals varied in size from 350 to 530 μm; the cuticle was smooth without sculpture; the claws were large. LM examination of whole mounts revealed the presence of males and females. Sex determination was based on the presence of gametes or eggs within gonads. Females contained several large eggs (Fig. 2A–B). Under a light microscope in lateral projection, the cloaca canal is visible, which is surrounded by a slight thickening of the cuticle (Fig. 2C). The absence of peribuccal sensory organs, a closed mouth and cloaca, and newly synthesized claws indicated that the individuals were in the simplex (molting) stage. In light microscopy preparations, the average body length of females was approximately 530 μm. The length of males was similar around 526 μm. Some individuals exhibited a protruding appendage at the caudal end between the legs of the 4th pair; these specimens measured approximately 350 μm long.

SCANNING ELECTRON MICROSCOPY (SEM). SEM examination showed three variants of the cloacal region structure in *H. crispae* (Figs. 2D–G; 3). Some individuals had a protruding cuticular cylinder at the site of the cloacal opening between the 4th pair of legs (Fig. 3A–D, F–G). The dimensions of this structure were approximately 34 μm in length and about 10 μm in diameter. In some specimens, the cloacal cylinder was partially extended; at the base of the incompletely protruded cylinder, semicircular folds were noticeable: an anterior fold and a pair of lateral folds, corresponding to oval-shaped cloacal plates (Fig. 3E–F). The diameter of the cylinder with basal folds was 23 μm. The folds at the base of the extended cloacal organ indicate a telescopic method of extension, a feature also characteristic of tardigrade limbs. In the studied specimens with extended cloacal cylinder, we observed compression of the posterior body region following the 3rd pair of legs, apparently due to muscle contraction. The apical end of the organ has a paired opening, around which ejaculated material was observed (Fig. 3C–D).

The identified female with eggs in the gonad (used for LM and subsequently for SEM) was found to have a diamond-shaped cloaca consisting of acutely angled triangular plates enclosing the opening (Fig. 2D–E). The dimensions of the diamond-shaped cloaca were 26.9 x 20.8 μm; height of the upper triangular plate was 8.5 μm, and the height of the lower pair of plates was 16.0 μm.

In addition, male individuals with an oval-shaped cloaca were observed. The dimensions of the oval cloaca were 21.7 x 13.8 μm; height of the upper oval plate was 7.98 μm, and the height of the lower pair of plates was 11.7 μm. In the specimens with an oval cloaca, we found a pair of distinct muscle attachment sites and a pair of prominent structures presumably corresponding to sensory papillae, located anterior to the cloaca (Fig. 2H–J).

CONFOCAL LASER SCANNING MICROSCOPY (CLSM). The two individuals (lacking distinct eggs) were examined for gonad structure using CLSM. The first specimen was at the active stage, as evidenced by separate macroplacoids in the pharynx and the presence of peribuccal sensory organs (not shown). The second specimen was in the simplex (molting) stage, with a double set of claws on the legs (Fig. 4A). Using DAPI staining, a sac-shaped male gonad filled with male reproductive cells at various developmental stages was identified on the dorsal side of both individuals, above the intestine (Fig. 4B). From the caudal end of the gonad to the posterior end of the body, two vasa deferentia extended around the gut (Fig. 4C–D, G). The gonoducts continued into a fusiform extension filled with sperm — the seminal vesicles

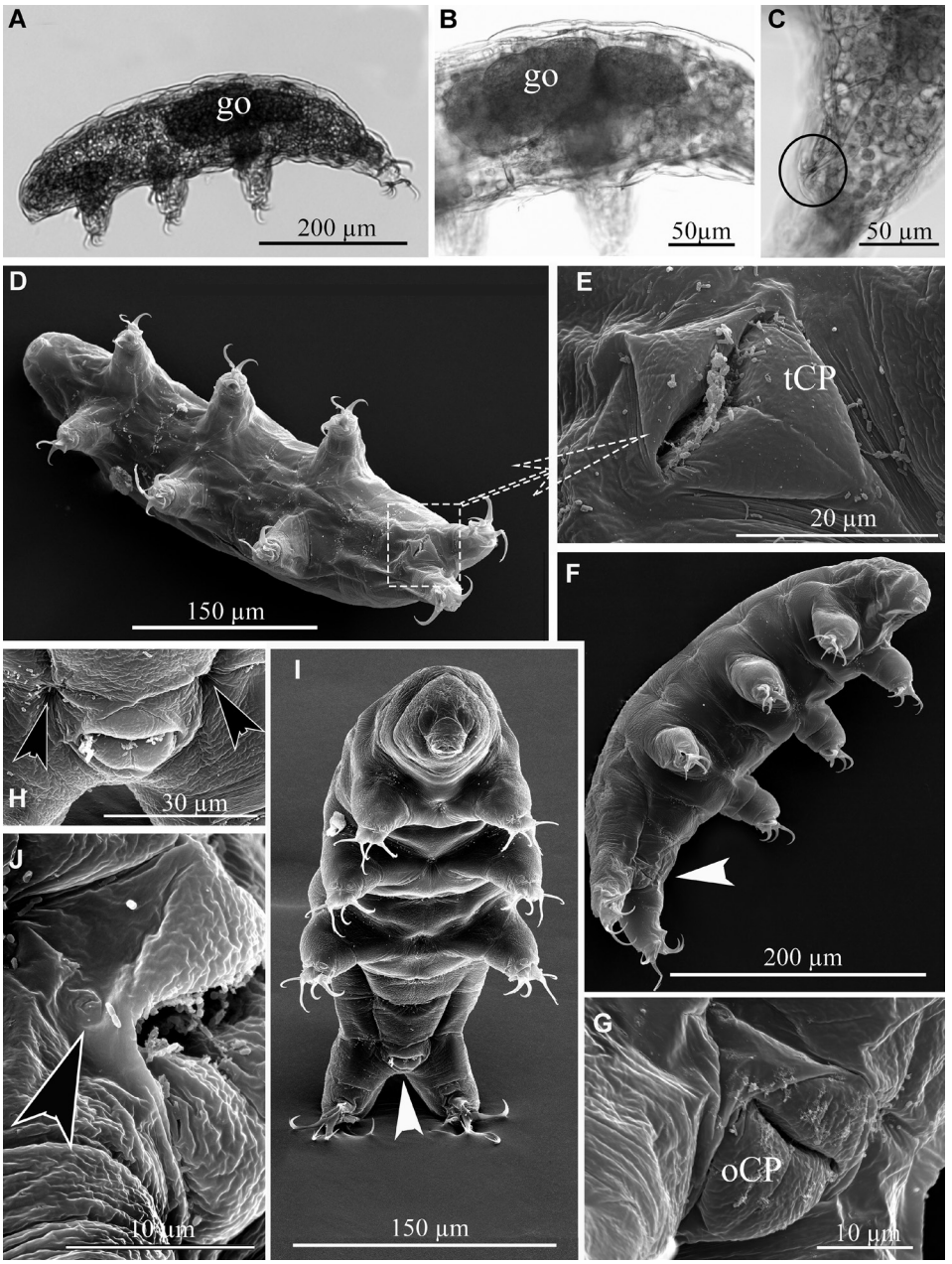

Fig. 2. General view of the *Halobiotus crispae* indicating external sexual dimorphism. A–E —female with eggs and diamond-shaped cloaca; F–J — presumable males with closed oval cloaca. A–C — LM; D–J — SEM. A — female of *Halobiotus crispae*, habitus, lateral view; B — part of the gonad with eggs; C — cloaca opening with a cuticular thickening (in a circle); D — female, habitus, ventral view; E — the ultrastructure of the female cloaca triangular plates from the fig. D; F — specimen 1 with the oval cloaca (arrowhead); presumable male, habitus, lateral view; G — the ultrastructure of the oval-shaped cloaca plates from the fig. F; H — specimen 2 with oval cloaca, ventral view; arrowheads indicating paired ventral intermediate attachment sites "g" of skeletal muscles; I — specimen 2 with the oval cloaca, habitus, anterioventral view; J — the sensory papilla located near the anterior plate of the oval cloaca (arrowhead).
Abbreviations: go — gonad; oCP — oval cloacal plates; tCP — triangular cloacal plates.

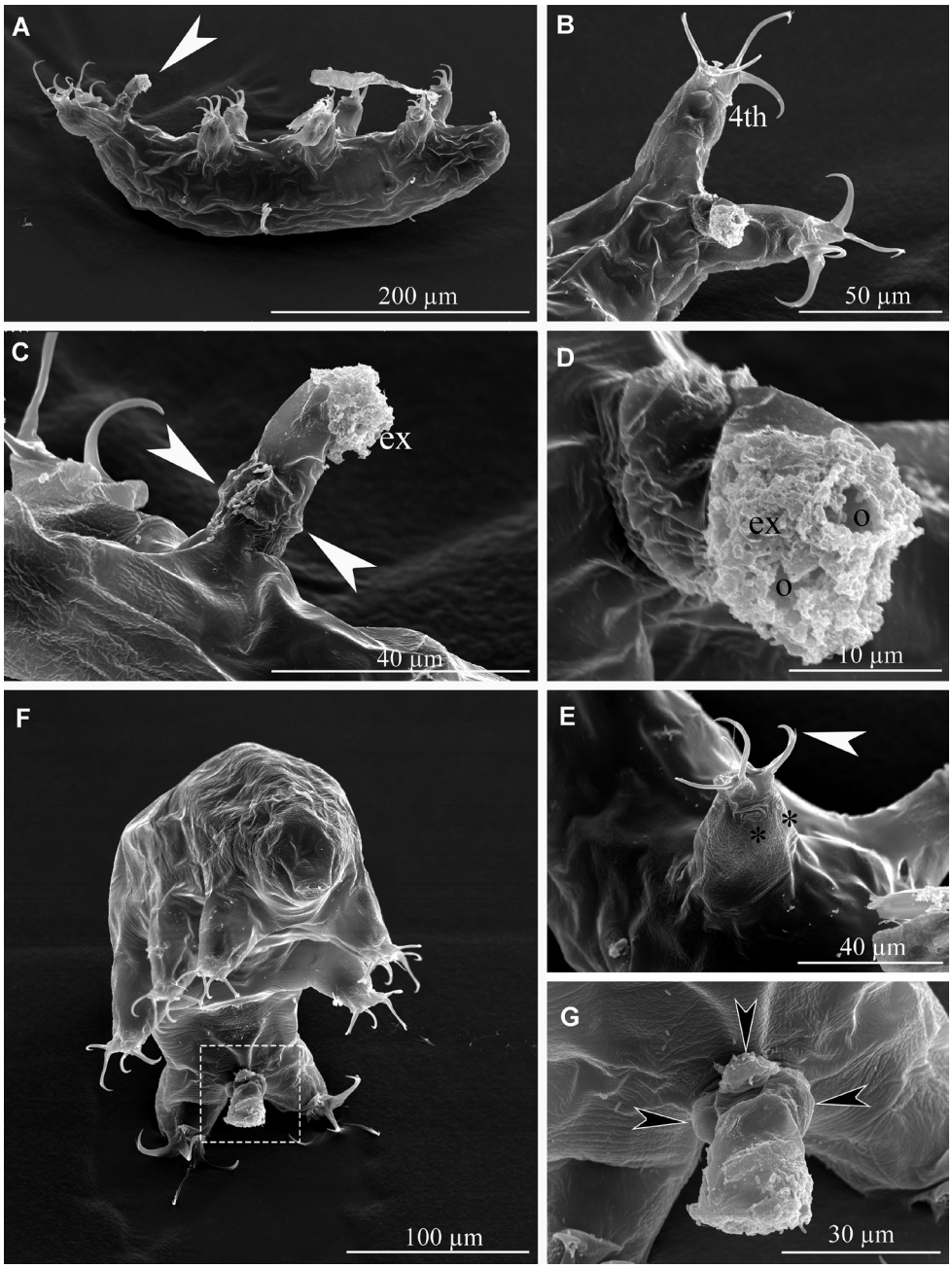

Fig. 3. The structure of the male cloacal organ of *Halobiotus crispae*, SEM. A — general view of the male with a protruding cloacal organ (arrow); lateral view, left side; B — the caudal end of the specimen on fig. A with 4th pair of legs and the pulled cloacal organ (arrow); C–D — details of the male cloacal organ in different projections; showing the basal folds (arrows) and ejaculated material around the opening; E — the 3rd pair of legs of the specimen on fig. A showing double claws, primary branches with accessory points and two sites of the muscle attachment (*) on the leg; F — the second specimen, habitus, anterio–ventral view; G — partially protruded cloacal organ of the specimen from F (in frame) with three cloacal plates at the base (arrowheads). Abbreviations: ex — ejaculated material; o — ejaculatory canal opening.

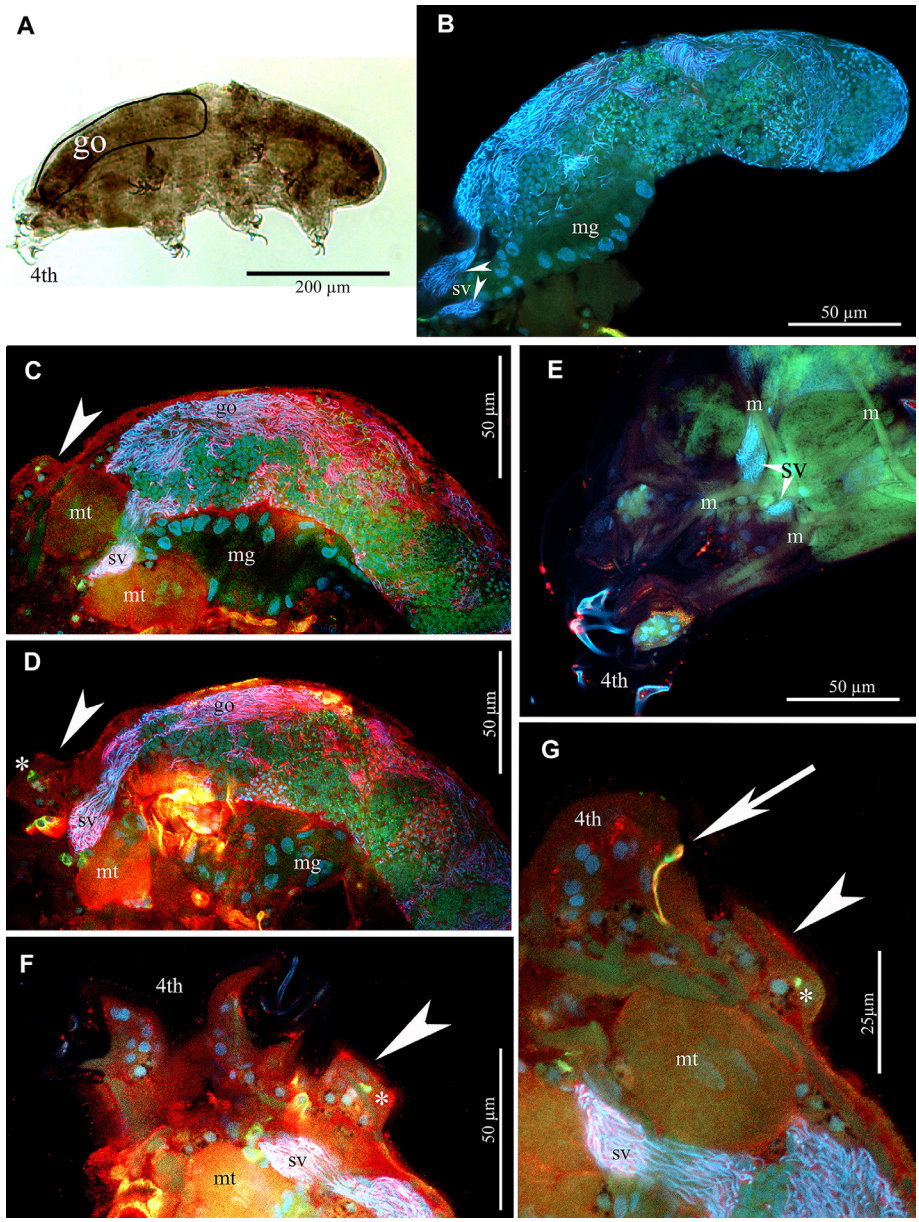

Fig. 4. The structure of the male gonad *Halobiotus crispae*; immunocytochemical staining, CLSM; DAPI in blue color; α-Tub in bright green; autofluorescence in other colors. A — general view of a male with the gonad, lateral view, anterior end facing right, LM; B — general view of the isolated gonad; two seminal vesicles are visible encircling the midgut, lateral view; C–D — posterior part of the male body; the right (C) and left (D) fusiform seminal vesicles are shown, one dorsal and a pair of ventral Malpighian tubules, part of the midgut and partially protruded part of the cloaca (arrowheads) with α-Tub-IR in the wall (asterisk); E — muscle bundles (autofluorescence) supporting the seminal vesicles; F — the posterior end of the body with the 4th pair of legs and partially extended cloacal organ (arrowhead), ventral view; seminal vesicle, Malpighian tubule and α-Tub-IR in the wall of cloacal cylinder (asterisk) are indicated; G — α-Tub-IR sensory ending in the 4th leg (arrow) and partially extended cloacal cylinder (arrowhead) with α-Tub-IR in the wall (asterisk). Abbreviations: go — gonad; m — muscle fiber; mg — midgut; mt — Malpighian tubules; sv — seminal vesicle; 4th — the 4th pair of legs.

(Fig. 4B–G). Each seminal vesicle was supported by at least two muscle bundles: one directed posteriorly and attached near the 4th pair of limbs, and the other oriented dorso-laterally and anchored to the inner cuticular surface (Fig. 4E). The right and left seminal vesicles were located above the ventral reservoirs of the Malpighian tubules, which had a common canal into the cloaca. The dorsal reservoir, located above the seminal vesicles, was connected to the cloaca via an independent canal. The apical part of the cloaca had cylindrical shape and protruded slightly above the surface (Fig. 4C, F).

Additionally, immunoreaction (IR) against α-Tub was detected in the cloacal cylinder and 4th pair of legs. Richly stained αTub-IR sensory neurites were observed in the fourth pair of legs, terminating on the ventral cuticular surface (Fig. 4G). The paired nerves terminated in noticeable bulbous expansions within the cuticle, consistent with ciliary endings. The α-Tub-IR was also detected in the wall of the cloacal cylinder (asterisks in Fig. 4D, F–G) at several optical sections.

Discussion

This study reports the first detection of *Halobiotus crispae* in the White Sea, confirmed by molecular studies. However, a large number of substitutions in ITS2 sequence of the White Sea isolate of *H. crispae* compared with other isolates of this species points on the beginning of a possible speciation process and likely driven by geographic isolation between populations in the White Sea and the Baffin Bay, the North Sea, the Baltic Sea.

Previously, it has been reported that another species of marine eutardigrade *Halobiotus appelloefi* (Richters, 1908) inhabited the White Sea in the vicinities of the White Sea Biological Station (WSBS MSU) (Petelina, Tchesunov, 1983). However, it was later synonymized with *Halobiotus stenostomus* (Richters, 1908) (Kaczmarek *et al*., 2015). *Halobiotus appelloefi* is currently regarded as *a nomen inquirendum* in the modern tardigrada taxonomy (Gasiorek *et al*., 2019; Degma *et al*., 2024). Nevertheless, due to the high morphological similarity among species of the genus, difficulties in identification caused by significant changes during cyclomorphosis and taxonomic confusion via synonymization with other species, the geographic range of *H. stenostomus* remains unresolved. Species identity of the White Sea populations has not been confirmed with molecular analysis and requires further verification (Biserova, Kuznetsova, 2012; Kaczmarek *et al*., 2015). Thus, to conclusively determine the number of *Halobiotus* species in the White Sea, a molecular analysis of the population near WSBS MSU is required.

Most of the animals described in our study were in the simplex (molting) stage, as evidenced by the absence of peribuccal sensory organs, closed mouth and cloaca, long claws, stripe-shaped placoids, double-layered cuticle, and a set of newly formed claws, which are clearly visible with LM and CLSM. One individual was in the active stage, indicated by the presence of macro- and microplacoids in the pharynx. Sexual maturation of *H. crispae* is thought to occur during the pseudosimplex 2 stage. Both the simplex and the active stages are characterized by fully developed gonads, allowing the sex of the animal to be determined (Kristensen, 1982). Thus, we discovered a population of *H. crispae* comprising sexually mature individuals of both sexes.

Sexual dimorphism in tardigrades remains poorly understood. In eutardigrades, minor differences in claw morphology have been documented in a few species (Bertolani, 1976, 1992, Suzuki, 2008). Among heterotardigrades, variations include gonopore morphology and position relative to the anus (Nelson *et al*., 2015; Altiero *et al*., 2018), and differences in the cuticle sculpture between males and females (Gąsiorek *et al*., 2020). Arthrotardigrades exhibit differences in the size of the clavae: males (58–70 μm) exhibit longer clavae (18–20 μm), while larger female (125–150 μm), possess shorter clavae (16–18 μm) (Kristensen, 1981).

In *H. crispae*, sexual dimorphism has not yet been shown (Kristensen, 1982), so the sex of individuals cannot be determined based on external morphology. However, according to Kristensen (1982), males of this species are smaller (body length: 295–585 μm) and have longer claws than females (whose body length is 510–666 μm). In our study, individuals with an extended cloacal cylinder measured approximately 350 μm in body length, which corresponds to the values characteristic for males of this species. However, other males which had their cloacal organ inverted were approximately the same size as the females.

At the active stage of the life cycle, the cloaca of *H. crispae* is a tri-lobed, slit-like structure. The cloacal opening is surrounded by cuticular plates, which close the opening at the pseudosimplex 1 and the simplex stages (Møbjerg *et al*., 2007; Halberg *et al.*, 2009b). Prior to our study, morphological characteristics distinguishing male and female cloacae were unknown. We identified differences in the size and shape of cloacae in *H. crispae*: males possess smaller oval-shaped cloaca, whereas females exhibit larger, diamond-shaped cloaca, with acute-triangular cloacal plates.

The cloacal cylinder observed in males is described here for the first time in tardigrades. In the individuals with protruded cloacal cylinder, the base diameter of the organ approximates that of closed putative male cloaca, while the terminal section of the cylinder is two times thinner. These dimensional differences support a telescopic protrusion mechanism similar to tardigrade limb extension. Particular structure of the muscular system indicates a possible extension of the cloacal cylinder. A detailed study of *H. crispae* musculature revealed the presence of two pairs of muscles associated with the cloaca (Halberg *et al.*, 2009a). These muscles originate from dorsal attachment sites and insert into the cloaca's dorsal wall. A pair of muscles located more caudally and forming the "P" attachment site is thought to be involved in the opening of the cloaca and extension of cloacal organ in male. The second pair, forming the so-called "U" site, forms a network of thin muscle fibers surrounding the cloaca (Halberg *et al.*, 2009a). Our findings demonstrate that at least two pairs of muscle bundles regulate the position of the seminal vesicles and, when contracted, appear to promote sperm ejaculation. To date, *H. crispae* is the only species with documented cloacal muscle networks. We identified the ventral muscle attachment sites anterior to the cloaca (Fig. 2H), which correspond to site "g" according to the classification of Halberg *et al*. (2009a). The two pairs of muscles supporting the seminal vesicles in male *H. crispae* demonstrated in our work correspond to muscles 7 iii extending into the 4th pair of legs and dorsoventral muscles 7 ii. For other tardigrade species, data on cloacal musculature is either limited (Dewel, Dewel, 1979; Marchioro *et al*., 2013), or is completely absent (Schmidt-Rhaesa, Kulessa, 2007; Halberg *et al*., 2013).

An important fact in the discussion of sexual reproduction strategies is the discovery of papillae in the region of cloaca (Fig. 2J) and the sensory dendrite ending in the 4th pair of legs (Fig. 4G). Previously, several nerves near the cloaca in *H. crispae* have been documented (Persson *et al.*, 2012): the nerves in the hind legs connecting leg ganglia in the distal part of the legs; a pair of nerves extending from the fourth ventral trunk ganglion and terminating near the cloaca. We have found paired α-Tub-IR nerves extending from the ganglia and terminating in cilia in the proximal part of the 4th legs and in the cloacal cylinder (asterisks in Fig. 4D, F–G). The sensory endings at the caudal end of *H. crispae* could be involved in reproduction and require more detailed investigation. The ultrastructure of the head sensory organs has been described in closely related species *H. stenostomus*; four types of ciliated receptors have been found (Biserova, Kuznetsova, 2012).

It is known that reproductive strategies vary significantly among representatives of different taxa of Tardigrada (Bertolani, 1983, 1990, 2001; Bertolani, Rebecchi, 1999). In marine taxa, cuticle-derived external pouches ("seminal receptacles") were found, in which sperm is stored between mating and oviposition (Jørgensen *et al*., 1999; Kristensen, Higgins, 1984). The process of ejaculation in males of limnoterrestrial eutardigrades has been recorded in four species that lay eggs in the exuvia (Sugiura, Matsumoto, 2021b): *Pseudobiotus megalonyx* Thulin, 1928*, Ursulinius nodosus* Murray, 1907*, Hypsibius convergens* Urbanowicz, 1925 and *Isohypsibius dastychi* Pilato, Bertolani et Binda, 1982; and in three species that lay eggs freely in the environment: *Paramacrobiotus* sp. isolate TYO, *Macrobiotus shonaicus* Stec, Arakawa et Michalczyk, 2018 and *Mesobiotus* sp. (Sugiura *et al*., 2019; Sugiura, Matsumoto, 2021b).

In *U. nodosus* and *H. convergens*, males ejaculate sperm into the cloacal opening of the exuvia with eggs. For *I. dastychi*, a male has been recorded to ejaculate while clasping the anterior end of female laying eggs in the exuvium with his caudal end (Sugiura, Matsumoto, 2021b). Among species that lay eggs freely in the environment, the fertilization process has been described in detail for three species of *Paramacrobiotus* sp. isolate TYO *Macrobiotus shonaicus* and *Mesobiotus* sp. (Sugiura *et al*., 2019; Sugiura, Matsumoto, 2021a). The authors identify 5 stages common

to these three species: Stage 1, tracking: a male tracks and focuses on a female; Stage 2, touching: a male touches a female's cloaca; Stage 3, standstill: a female stops moving until a male ejaculates; Stage 4, ejaculation: a male curls his tail end and ejects sperm into the cloaca at close range; Stage 5, contraction: a female contracts the ventral side after ejaculation to capture sperm deposited in the external environment in the immediate vicinity of the cloaca (Sugiura *et al*., 2019). Mating behavior of *H. crispae* hasn't been recorded yet (Sugiura, Matsumoto, 2021b). Seminal receptacles were also not observed in this species (Kristensen, 1982). New results including the discovery of the cloacal cylinder, sensory endings and specialized musculature at the posterior end of males suggest complex reproductive behavior of *H. crispae*. We assume that sexual dimorphism in *H. crispae* will be discovered throughout its geographic range.

Conclusions

Our study reveals new data on the reproductive biology of a tardigrade species *Halobiotus crispae* including sexual dimorphism and insights into fertilization mechanisms. Key discoveries include: 1) cloacal cylinder in males: males possess a protrusible cloacal cylinder, likely functioning as a copulatory organ. Its extension due to contraction of caudal and cloacal muscles suggests that it is used for internal fertilization, rarely observed in eutardigrades. This observation widens our knowledge of the reproductive strategies in tardigrades; 2) sexual dimorphism: distinct differences in cloacal morphology were observed. Males have oval-shaped and smaller cloaca with smaller size of cloacal plates; females have larger, diamond-shaped cloaca, with acute-triangular cloacal plates. This represents the first documented case of external sexual dimorphism in eutardigrades; 3) sensory organs and reproductive behavior: sensory structures near the cloacal plates and on the fourth pair of legs imply neural regulation of mating, potentially guiding copulatory interactions. Our study highlights the need for the detailed investigation of tardigrade reproduction, particularly to clarify the prevalence and mechanisms of internal fertilization

Compliance with ethical standards

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

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