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Sexual reproductive biology of twelve species of rotifers in the genera: *Brachionus, Cephalodella, Collotheca, Epiphanes, Filinia, Lecane,* and *Trichocerca*

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ABSTRACT
We provide descriptions of the sexual reproductive biology of 12 species of rotifers from seven families and seven genera: *Brachionus angularis, B. araceliae, B. ibericus, B. quadridentatus* (Brachionidae); *Cephalodella catellina* (Notommatidae)*; Collotheca ornata* (Collothecidae); *Epiphanes brachionus* (Epiphanidae), *Filinia novaezealandiae* (Trochosphaeridae); *Lecane nana, L. leontina, L. bulla* (Gosse 1851) (Lecanidae); and *Trichocerca stylata* (Trichocercidae). Data include: (a) video-recordings of 10 of the 12 species (the exceptions are two common species, *B. angularis* and *B. ibericus*), (b) scanning electron micrographs of *B. araceliae*, *B. ibericus*, *C. catellina,* and *E. brachionus* females, (c) light micrographs of *C. catellina, C. ornata, F. novaezealandiae*, *L. bulla*, *L. leontina*, *L. nana,* and *T. stylata* diapausing embryos, males, and unfertilized sexual eggs. This study challenges the common perception that mating behavior consists of five steps, and provides: (a) the first documentation of sexual cannibalism in *C. catellina*, (b) the first description of the sexual reproductive behaviors of *C. ornata*, *L. bulla, L. leontina, L. nana*, and *T. stylata*, (c) the first description of the males of *F. novaezealandiae*, *L. leontina, L. nana,* and *T. stylata*. Moreover, this study includes analyses of morphological and behavioral variation among males of the seven families studied.

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Introduction

Invertebrates exhibit a great diversity in their reproductive strategies. Asexual reproduction can lead to fast population growth, but it does not give rise to the genetic diversity inherent in sexual reproductive processes (Pouchkina-Stantcheva et al. 2007). In some taxa, sexual reproduction results in the concomitant production of diapausing embryos or other types of resting stages (Oehlmann & Schulte-Oehlmann 2003). For example, in cyclical

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parthenogenetic monogonont rotifers fertilization of sexual females during the sexual (mictic) phase results in the production of diapausing embryos (also known as resting eggs or cysts). These diapausing embryos provide recombinant genotypes as well as a mechanism for dispersal in time and space (Walsh et al. 2017).

Induction of mictic reproduction in rotifers is a highly adaptive mechanism of great ecological relevance and has been extensively studied in species of the genus *Brachionus*. This genus is widely used in aquaculture, and sexual reproductive behaviors and high levels of diapausing embryos production are often noted (Rico-Martinez & Snell 1997). In *Brachionus* and other monogonont rotifers, the shift from asexual to sexual reproduction and the production of males occurs in response to environmental cues including: (a) changes in photoperiod, (b) crowding, or (c) diet (α-tocopherol in *Asplanchna*) (Gilbert & Williamson 1983). Sexual reproductive behavior of rotifers includes conspecific recognition that is mediated through chemoreceptors (Rico-Martinez & Snell 1997).

Mating behaviors of monogonont rotifers typically consists of five steps: (a) encounter, (b) circling, (c) coronal localization, (d) sperm transfer, and (e) dissociation (Wallace et al. 2006). While almost all studied species follow this pattern to a great extent, several variations have been noted (Rico-Martinez & Snell 1997; Rico-Martinez & Walsh 2013). Among these variants are observations of several males copulating simultaneously the same female (pers. obs. by Rico-Martínez; Walsh), egg-guarding behavior (Schröder 2003; Schröder & Walsh 2010), and necrophilia (Snell et al. 1995).

Despite the importance of sexual reproduction in this phylum, there are still many species for which the male has not yet been observed or described (Walsh et al. 2017), and behaviors associated with mating are similarly lacking. While progress has been made in understanding the role of sexual and reproductive behaviors in monogonont rotifers, some questions remain unanswered. (a) How widespread is the “typical” pattern of mating behavior among rotifer species? (b) Is our current understanding of mating behavior an artifact arising from the overwhelming bias of data on brachionid mating behavior and the scarcity of data on other families? (c) How common are unique behaviors among taxa? (d) Are traits of the male a result of evolutionary or ecological forces? Consequently, it is of interest to study sexual reproduction of a wider array of rotifer species to gain a better understanding of patterns and processes that comprise their complex mating behaviors and morphological adaptations.

Here we provide a detailed description of the sexual reproductive behaviors and morphology of males of 12 monogonont species representing diversity of females to begin to address these and related questions. In addition we provide the first documentation of cannibalism of larval females in *Collotheca ornata* (Ehrenberg 1830) and sexual cannibalism of males following mating in *Cephalodella catellina* (Müller 1786).

Materials and methods

We collected 10 species from the following sources in Mexico: (a) *Cephalodella catellina* from a small, temporary pond near the municipality of San Francisco De Los Romos, in Aguascalientes (GPS coordinates: 22°02.704 N, 102°27.467 W), (b) *Brachionus araceliae* Silva-Briano, Galván-De la Rosa, Pérez-Legaspi and Rico-Martínez 2007 and *Filinia novaezealandiae* (Shiel and Sanoamuang 1993) in Zacatecas (21°25.2 N, 102°40.57 W and 21°29.35 N, 102°48.22 W, respectively), (c) *Epiphanes brachionus* (Ehrenberg 1837) from

Presa Tolimique, Ejido El Ocote, Aguascalientes (21°47.09 N, 102°1.04 W), (d) *Brachionus angularis* Gosse 1851 and *B. quadridentatus* (Hermann 1783) from the wastewater treatment plant pond on the campus of the Universidad Autónoma de Aguascalientes (21°53.10 N, 102°28.54 W), (e) *Brachionus ibericus* Ciros-Pérez et al. 2001; *Trichocerca stylata* (Gosse 1851) and *Lecane bulla* (Gosse 1851) from a mangrove in Cancún, Quintana Roo (21°11.43 N, 86°49.0 W), and (f) *Lecane leontina* (Turner 1892) from a temporary pond near the La-Salle University of Cancún (21°03.122 N, 86°50.4242 W). All of these species were collected with a Wisconsin type, 45 μm mesh planktonic net. Identification was made using keys from Koste (1978), Nogrady and Pourriot (1995), and Segers (1995). *Brachionus angularis*, *B. araceliae*, *B. quadridentatus, C. catellina, E. brachionus*, *F. novaezealandiae, L. bulla*, and *L. leontina* were kept in 24-well polystyrene plates with a final volume of 1 ml of EPA medium (Weber 1993). Originally 1 ml of the water from the corresponding reservoir (previously filter through a 45 μm mesh) was added to obtain a 1:1 solution with EPA medium. The rotifers were fed with the green algae *Nanochloropsis oculata* Hibberd 1981 (UTEX Culture Collection of Algae, strain LB2164) grown in Bold Basal Medium (Nichols 1973). After an accumulation period of at least two months, the species were eventually transferred to 100% EPA medium and cultured at 25 °C in a bioclimatic chamber with a photoperiod of 18:6 h Light:Dark. Species were cultured for two months before initiating experiments in most cases.

Lecane nana (Murray 1913) was collected from Hueco Tanks State Park and Historic Site, El Paso Co., TX, USA (31°55.24 N, 106°02.28 W). This species was initially cultured in modified MBL medium (Stemberger 1981) and fed *Cryptomonas erosa* Ehrenberg 1832. Later it was transported to Aguascalientes where it was kept under same conditions as described above for *L. bulla* and other freshwater species.

We conducted morphometric analyses of males, females, parthenogenetic eggs, and diapausing embryos for all species listed above, and obtained the male to female length ratio by dividing the mean length of the male by the mean length of the female for each species. We obtained videos and photomicrographs with a Leica DMLS light microscope equipped with an Infinity 3 camera and Infinity Capture 4.6.0 software (Lumenera Co., Ottawa, ON, Canada) and a Zeiss Axioscope compound microscope, using AxioVision software (SE64Rel 4.8 Inc. 2003). We obtained data on sexual reproductive behavior through the analysis of the video recordings. We made scanning electron micrographs with a JEOL LV 5900 microscope. Extraction of trophi was based on the protocol of Guerrero-Jiménez et al. (2015). Drawings of males were made based on light micrographs by tracing the contour of the animal on vellum paper and followed by digitization.

Collotheca ornata hatched from rehydrated sediments collected from a temporary wetland behind an artificial dam in Mescalero Canyon, Hueco Tanks State Park and Historic Site, El Paso Co., TX, USA (31°55.12 N, 106′02.24 W). EPA medium (Weber 1993) was used to rehydrate sediments. Rotifers were cultured in modified MBL (Stemberger 1981) and fed with the algae *Chlamydomonas reinhardtii* Dangeard 1888 (UTEX strain 90), *Chlorella vulgaris* Berijerinck 1890 (UTEX strain 30), and *Cryptomonas erosa* Ehrenberg 1832. To observe mating behaviors, males and mictic females of *C. ornata* were isolated and placed in a drop of MBL on a slide. We recorded mating behaviors using a modified Logitech web camera attached to a Leica Wild M10 dissecting scope and AMCap software 9.21 (© 2004–2012, Noël Danjou). The analyses of mating behaviors were based on 48 trials; ≤15 ($n = 9$), 20 ($n = 6$), 30 ($n = 3$), and 60 ($n = 30$) min trials. Fertilization after copulation was not confirmed because sperm were not visible within the females' body. To measure male swimming speed, single males were placed in a small drop of MBL on a stage micrometer without a coverslip. All observations were made at room temperature (approximately 21 °C). Male swimming behavior (*n* = 16, 1–4 observations per male) was video-recorded (2 min trials) and speed (mm/s) was determined by calculating the linear swimming distance. To determine male life span, unfertilized mictic eggs were isolated and neonate males were collected every 15 min, placed into individual culture dishes, and monitored for survival every 30 min until death.

Results

Sexual reproductive behaviors of Brachionus angularis *(Family Brachionidae)*

The males of *B. angularis* were very active, fast swimmers and constantly interacted with multiple females. The average copulation time in *B. angularis* was 25.3 ± 13.1 s (*n* = 10; Table 3). Copulation occurred at the corona. Males tended to copulate with older females with reduced mobility. The mean copulation time was the shortest recorded for all species we examined.

Sexual reproductive behaviors of B. araceliae *(Family Brachionidae)*

Sexual reproductive behaviors of *B. araceliae* are identical to those described for other *Brachionus* species (e.g. *B. calyciflorus*, *B. quadridentatus*, and *B. plicatilis* Müller 1786). The mean copulation time was 57.7 ± 16.9 s (*n* = 3; Table 3). Copulation occurred at the corona. We observed two males copulating simultaneously the same female (see supplementary video 1). Figure 1 shows scanning electron photomicrographs of an amictic female (Figure 1(A)), a mictic female carrying a diapausing embryo (Figure 1(B), (D)), a mictic female

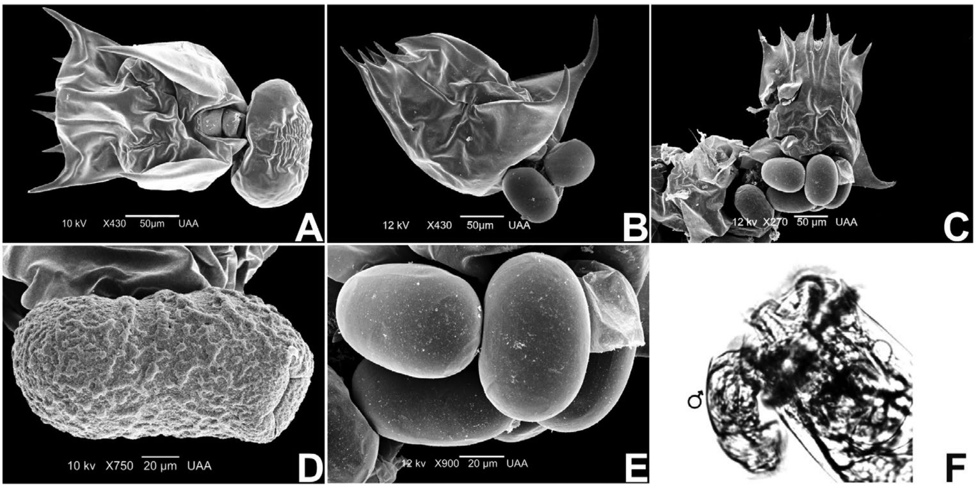

Figure 1. *Brachionus araceliae.*
Notes: (A) females with an amictic egg, habitus in dorsal view, (B and C) females with unfertilized mictic eggs, (D) resting egg, (E) unfertilized mictic eggs, (F) male copulating a female at the corona.

with an unfertilized egg (Figure 1(C), (E)), a male (Figure 1(F)), and a still image captured from supplemental video 3 showing the copulation site (Figure 1(F)).

Sexual reproductive behaviors of B. ibericus *(Family Brachionidae)*

Sexual reproductive behaviors of *B. ibericus* strain Cancún are similar to the mating behaviors described for other species of the genus *Brachionus*. Scanning electronic photomicrographs show details of an amictic female (Figure 2(A), (B)). The male corona has long, thick

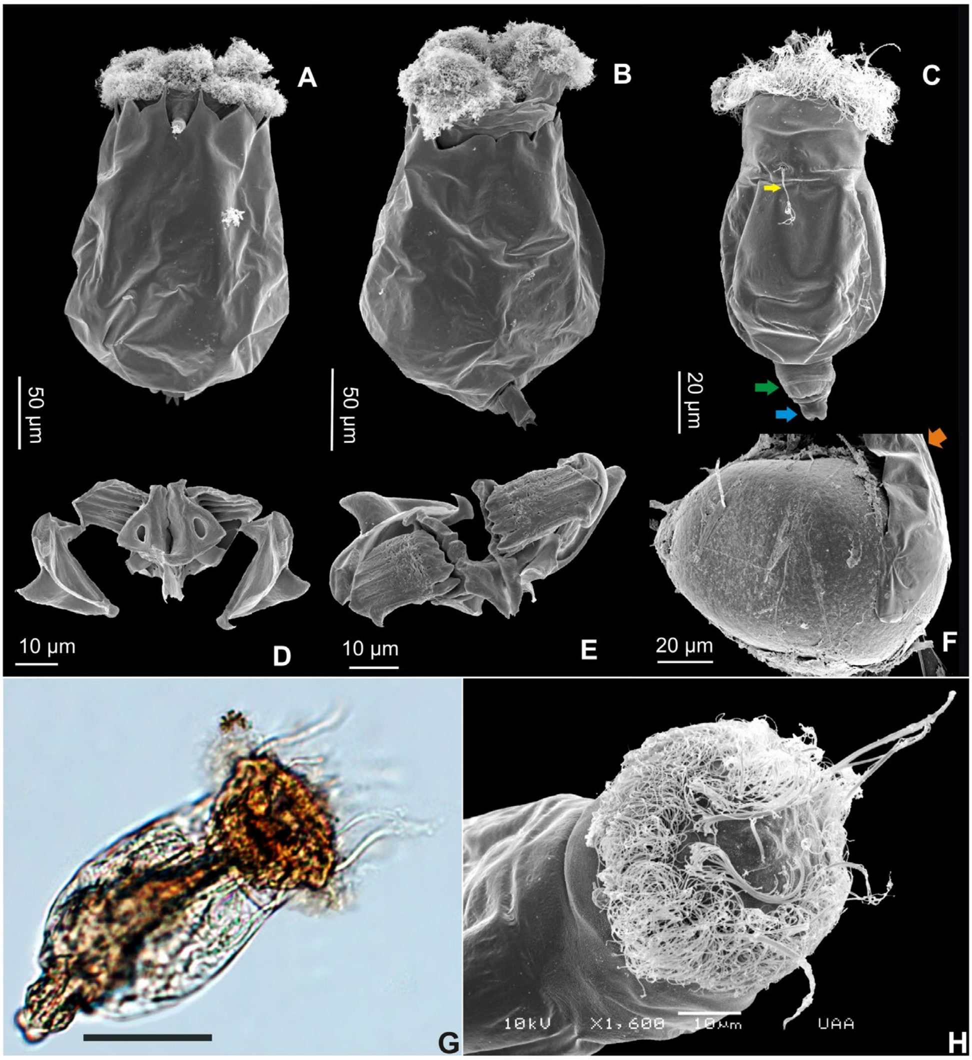

Figure 2. *Brachionus ibericus* strain Cancún.
Notes: (A) habitus of amictic females in dorsal view, (B) amictic female in ventral view, (C) male, the yellow arrow indicates the dorsal antenna, the green arrow indicates the penis, and the blue arrow indicates the foot, (D and E) the trophi, (F) the resting egg remains inside the lorica (brown arrow indicate the lorica of the rotifer), (G and H) details of the males corona, note the long, thick cilia.

cilia (Figure 2(C), (G), (H)). The trophi is shown in Figure 2(D) and (E). The resting eggs are shown in Figure 2(F) and, as seen in the figure, they remain inside the female's lorica.

Additions to the sexual reproductive behaviors of **B. quadridentatus** *(Family Brachionidae)*

Sexual reproductive behaviors of *B. quadridentatus* were described by Díaz et al. (2006) and were confirmed in our observations of this species. Copulation occurred at corona and foot aperture. In addition, five males copulating simultaneously with a single female at the corona is shown in supplementary video 2 (00:00 to 01:10).

Sexual reproductive behaviors and sexual cannibalism in **Cephalodella catellina** *(Family Notommatidae)*

We observed that females of *C. catellina* consumed their mates after copulation. This behavior is shown in two video-recordings (supplementary videos 3 and 4). Cannibalistic behavior started when the male made contact with the female and attached to the posterior part of the female (near the foot) with the help of its dorsal antenna (supplementary video 3). Then female appeared to attack the male to dislodge the male. However, the male was able to complete copulation in all trials. Copulation ended when the female detached from the male and started flicking her toes at the male (e.g. around 21 times until its death: 1:08 to 4:04 min, supplementary video 4). Afterwards the female ingested the male (see supplementary video 4: during this range of time: 1:08 to 4:04 min). Attachment was observed four times, and copulation followed by female ingestion of the male was observed twice (Table 2). Figure 3 shows a *C. catellina* amictic female (Figure 3(A), (D)), the resting egg (Figure 3(B), (F)), the amictic egg (Figure 3(C)), the trophi (Figure 3(E)), and the male (Figure 3(G)). Table 1 shows the morphometric analyses of males, females, parthenogenetic eggs, and diapausing eggs of *C. catellina*.

Table 1. Measurements of females and males in 11 rotifer species: BIB = *Brachionus ibericus*, BAR = *B. araceliae*, BQU = *B. quadridentatus*, FNO = *Filina novaezealandiae*, EBR = *Epiphanes brachionus*, CCA = *Cephalodella catellina*, LNA = *Lecane nana*, LLE = *L. leontina*, LBU = *L. bulla*, TST = *Trichocera stylata*, CO = *Collotheca ornata*. The mark + indicates data obtained from Silva-Briano (2007) and * data obtained from Díaz et al. (2006).

	Female µm (Mean ± SD)			Male µm (Mean ± SD)			Male to female length ratio
	Length	Width	*n*	Length	Width	*n*	
BIB	225.0 ± 38.8	173.4 ± 28.9	20	91.89 ± 6.3	48.6 ± 9.5	20	0.41
BAR+	637.5 ± 54.4	229 ± 22.0	20	159.4 ± 26.1	66.2 ± 14.0	20	0.25
BQU*	163.0 ± 27.3	73.6 ± 9.4	20	53.8 ± 9.3	33.6 ± 6.1	40	0.33
FNO	164.9 ± 11.8	89.9 ± 10.7	3	47.2	32.4	1	0.29
EBR	210	132	1	179 ± 7.4	68.4 ± 7.4	4	0.61
CCA	113.3 ± 9.1	17.6 ± 1.7	7	101.2 ± 15.2	33.19 ±6.2	2	0.89
LNA	172.6 ± 20.0	77.7 ± 3. 8	3	137.9 ± 19.3	38.25 ± 1.7	4	0.80
LLE	182.4 ± 11.7	132,1 ± 4.2	22	100.3 ± 6.1	56.24 ± 2.9	21	0.55
LBU	130.0 ± 8.4	92.4 ± 6.5	12	114.3 ± 7.5	38.0 ± 7.5	11	0.88
TST	83.5 ± 10.1	50.2 ± 9.3	21	43.8 ± 2.3	31.03 ± 1.9	3	0.52
CO	413.9 ± 31.3	nd	13	92.6 ± 31.3	18.2 ± 2.9	13	0.22

nd = No data.

Table 2. Morphometric measurements of asexual and sexual eggs in 10 rotifer species. BIB = *Brachionus ibericus*, BAR = *B. araceliae*, BQU== *B. quadridentatus*, FNO = *Filinia novaezealandiae* EBR = *Epiphanes brachionus*, CCA = *Cephalodella catellina*, LNA = *Lecane nana*, LLE = *L. leontina*, LBU = *L. bulla*, CO = *Collotheca ornata*.

Diapausing embryos µm (Mean ± SD)				Amictic eggs µm (Mean ± SD)			Unfertilized mictic eggs µm (Mean ± SD)		
	Length	Width	*n*	Length	Width	*n*	Length	Width	*n*
BIB	68.6 ± 4.6	82.1 ± 4.0	20	127.0 ± 10.8	99.3 ± 6.7	20	66. 3 ± 9.3	60.2 ± 4.0	20
BAR	140	70	1	110.2	80.5	1	64.7 ± 7.1	45.2 ± 3.7	4
BQU*	146.6 ± 9.2	91.6 ± 4.7	11	129.5 ± 10.1	81.8 ± 9.9	20	–	–	–
FNO	82.5	64.9	1	47.2	47.2	1	36.6 ± 2.47	26.5 ± 0.8	4
EBR	92.2 ± 4.8	71 ± 7.5	14	57.2	51.2	1	128.3 ± 12.3	87.7 ±7.1	10
CCA	68.8	51.5	1	58.7	41.8	1	–	–	–
LNA	68.6 ± 4.6	82.1 ± 4.0	7	–	–	–	–	–	–
LLE	109.8 ± 8.7	45.8 ± 5.3	5	114.9 ± 3.1	84.8 ± 2.4	4	78.1 ± 4.6	63.9 ± 3.5	4
LBU	70.5 ± 7.1	61.9 ± 6.8	5	79.9 ± 7.5	66.4 ± 6.4	20	53.8 ± 3.5	55.25 ± 4.3	4
CO	53.3 ± 2.0	42.2 ± 3.4	10	52.8 ± 4.8	31.8 ± 3.4	12	46 ± 7.3	27.8 ± 2.5	42

Notes: *indicates data were obtained from Díaz et al. (2006); – = No data.

Table 3. Summary of mating behaviors in 12 rotifer species. BIB = *Brachionus ibericus*, BAR = *B. araceliae*, BQU = *B. quadridentatus*, BAN = *B. angularis*, FNO = *Filinia novaezealandiae*, EBR = *Epiphanes brachionus*, CCA = *Cephalodella catellina*, LNA = *Lecane nana*, LLE = *L. leontina*, LBU = *L. bulla*, TST = *Trichocerca stylata*, CO = *Collotheca ornata*.

	Sites of copulation	Resting egg position	Duration of copulation (s)	Males that copulated with a female (*N*)	Male swimming speed (mm/s)
BIB	Corona	Inside	–	1	–
BAR	Corona	Outsde	50.0 ($n = 1$)	2	–
BQU*	Corona and Foot Opening (95%)*	Outside	71.4 ± 11.6 ($n = 16$)	5	9.7 ± 1.3 ($n = 10$)*
BAN	Corona	Outside	25.3 ± 13.1 ($n = 10$)	1	–
FNO	Foot opening	Inside	–	1	–
EBR	Corona	Outside	–	2	–
CCA	Foot opening	Outside	38.0 ± 15.0 ($n = 3$)	1	–
LNA	Corona, juncture of ventral and dorsal plates at mid-region of lorica	Outside	108.0	1	–
LLE	Corona and cloaca	Outside	45.5 ± 12.0 ($n = 2$)	1	–
LBU+	At juncture of ventral and dorsal plates at mid-region of lorica	Inside	1081.2 ± 132.0 ($n = 6$)	1	0.4 ± 0.2 ($n = 10$)
TST	Foot opening	–	303.0 ± 12.0 ($n = 2$)	1	–
CO	Neck region ($n = 1$) Coronal lobe ($n = 6$) Not determined ($n = 1$)	Outside	416. 2 (36–1910) ($n = 8$)	1	0.032 ± 0.007 ($n = 16$)

The + indicates data were obtained from Segers and Rico-Martínez (2000).
*refers to data obtained from Díaz et al. (2006); – = No data.

Sexual reproductive behaviors of Collotheca ornata *(Family Collothecidae)*

In *Collotheca ornata* mating behaviors included encounter, circling, coronal localization, copulation, and dissociation. However, 29.1% of males started coronal localization upon encountering the female without exhibiting the typical circling behavior. In mating trials, 70.8% (34 of 48 trials) of males started circling behavior after encountering female. Of circling attempts, 85.3% resulted in coronal localization. For coronal localization, males

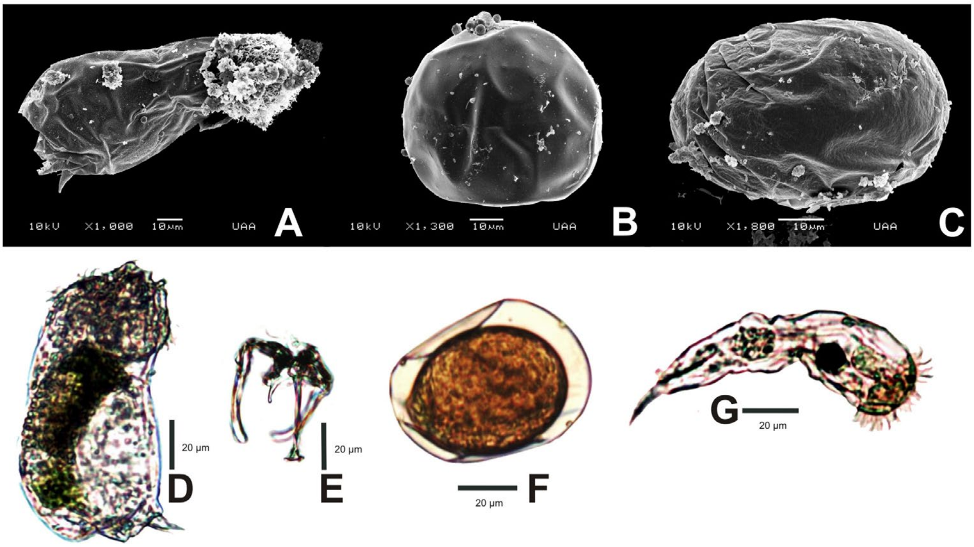

Figure 3. *Cephalodella catellina*.
Notes: (A) amictic females habitus, (B) resting eggs, (C) amictic egg, (D) amictic females lateral view, (E) trophi type C, (F) image of resting eggs obtained by light microscopy, (G) male habitus.

used only their corona in 77% of trials (37 out of 48; Figure 4) and both their corona and penis in 13.9% of trial (6 out of 48). Copulation was achieved in 18.6% of coronal localization attempts (Table 1). Mean duration of circling was 14.3 s (range: 2 – 49.6 s), duration of coronal localization was 16 s (range: 0.8 –31.2 s), and duration of coronal localization by male using the penis was 8.5 s (range: 4 – 14 s). Some of these behaviors can be seen in supplementary video 5. For instance, coronal localization starts at 00:29 min after circling and copulation starts at 00:37 min. Copulation takes place at the neck of the female and stops at 01:37 min. Next, the female retracts her corona at 01:51 min and the male leaves. Morphological details of the female (Figure 4(A), (B)), male (Figure 4(C), (D)) and eggs (Figure 4(E)) are shown. Figure 4(F) shows coronal localization. Morphological data are given in Tables 1 and 2.

Sexual reproductive behaviors of Epiphanes brachionus *(Family Epiphanidae)*

Males were very active and they contacted the corona and the mid-region of the female's lorica several times during mating trials. The copulation site was the female's corona. The sexual reproductive behaviors of *E. brachionus* are shown in supplementary video 2 from 02:27 to 03:46. As seen in the video, it is evident that the male has the typical conical shape of all males observed so far in the genera *Epiphanes* and *Brachionus*. In supplementary video 2 (at 2:40 min), two males contacted one female simultaneously. In Figure 5(A), (B) morphological details of female and male of *E. brachionus* are shown.

Figure 4. *Collotheca ornata*.
Notes: (A) female habitus, (B) female with amictic egg, (C) mictic eggs and neonate male in gelatinous sheath of female, (D) male, (E) resting egg, (F) coronal localization by male.

Sexual reproductive behaviors of Filinia novaezealandiae *(Family Trochosphaeridae)*

Sexual reproductive behaviors of *F. novaezealandiae* including mating attempts and copulation near the central posterior seta of the female are shown in supplementary video 2

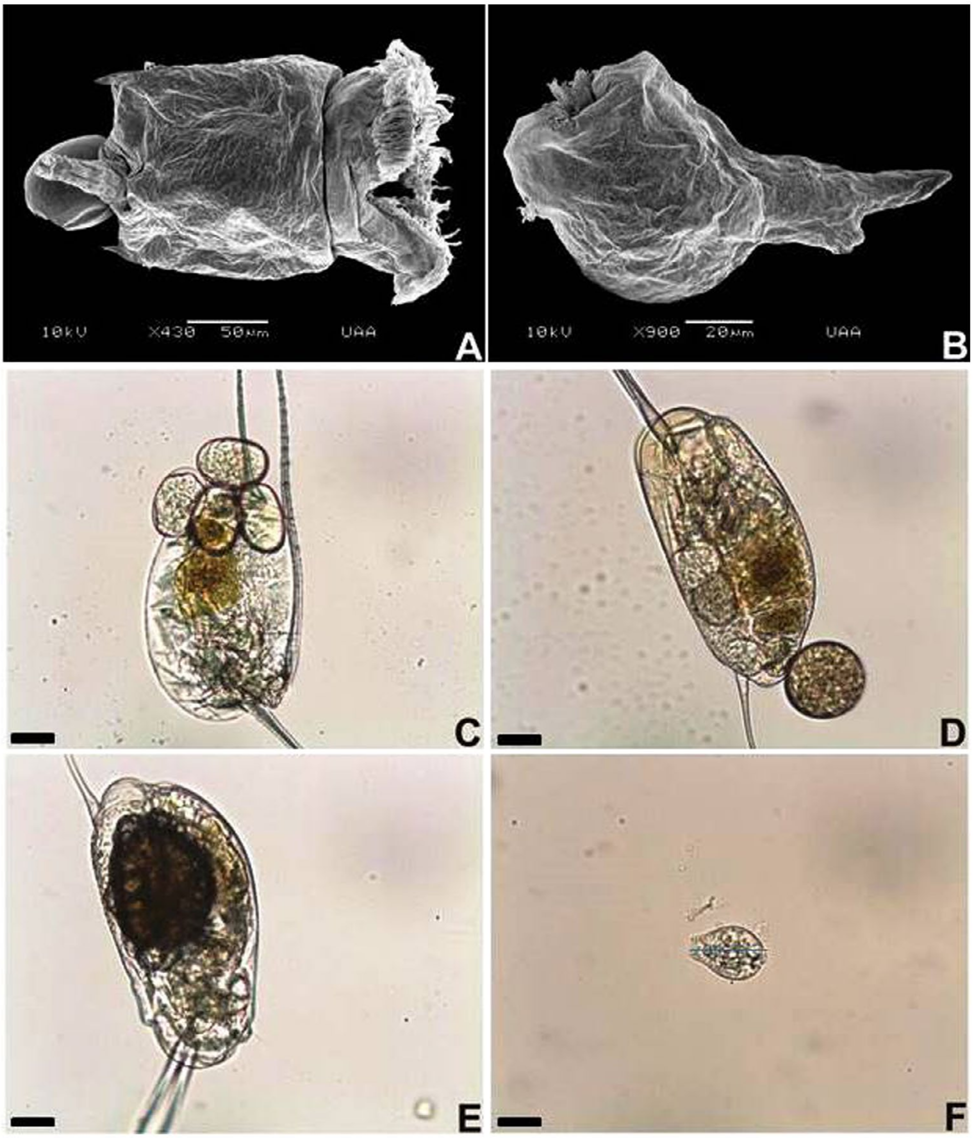

Figure 5. *Epiphanes brachionus* and *Filinia novaezealandiae*.
Notes: (A) habitus of amictic female of *E. brachionus* with one amictic egg, (B) males of *E. brachionus*, (C) amictic female of *F. novaezealandiae* with four unfertilized mictic eggs, (D) females of *F. novaezealandiae* with one amictic egg, (E) female with a resting egg inside the body, (F) the male of *F. novaezealandiae*. Bars = 25 μm.

(01:10 to 02:25). As can be seen in the video, the male shows great mobility. A diapausing embryo can also be seen developing inside of some females, while unfertilized mictic eggs are being carried by others. Male embryonic development is shown in supplementary video 2 from 01:52 to 02:15 min. Morphometric measurement of females and eggs (n = 3) and the male (n = 1) are shown in Tables 1 and 2. The diapausing embryo is completely round with a net-like ornamentation that differs from the amictic egg, which has an ovoid shape. The male's body is saccate and widest at the center. Details of female and male morphology are shown in Figure 5(C)–(F).

Additions to the sexual reproductive behavior of Lecane bulla *(Family Lecanidae)*

In *L. bulla*, copulation occurs at the juncture of the dorsal and ventral plates (Segers & Rico-Martínez 2000). In our study duration of copulation in *L. bulla* was 18.0 ± 2.2 min. Sperm transfer was slow (supplementary video 6), which may provide an explanation for the long copulation time recorded for this species (Table 3). Morphological details of the female, male, and amictic, mictic and diapausing eggs are shown in Figure 6(A)–(F).

Sexual reproductive behavior of Lecane leontina *(Family Lecanidae)*

Males of *L. leontina* move slowly and make gyratory movements, adhering to the substratum at the bottom of the culture dish. We observed groups of four males, sometimes joined together by their feet gyrating along their own axis. A group of five males attempted to copulate with an empty eggshell near a female, but they did not try to copulate with the live female. This behavior lasted up to 100 s. In another recording, we observed two males attempting to copulate with an empty eggshell, and again a nearby female was ignored for up to 100 s. Males copulated with females through the cloaca and the coronal aperture. When copulation was through the cloaca, the male first attached itself near the trapezoidal foot pseudosegment of the female and the male penis slid through it toward the cloaca. The male reached the cloaca only after approaching the dorsal plate of the female thus avoiding being disrupted by the female's toe movements (which are constant and gyratory during copulation). In supplementary video 7 this mating behavior is observed twice (first instance: 00:39 – 00:55; second instance: 00:56 – 1:11 min). We surmise that copulation may be easier for the male when it is performed through the coronal aperture, because the cuticle is soft at the juncture between the ventral and dorsal plates of the female's lorica. Copulation lasted longer at the corona than at the cloaca (corona: 45.5 ± 12.0 s ($n = 2$); cloaca, 13.5 s; ($n = 1$)). Sexual reproductive behaviors of this species are shown in supplementary video 7. The details of the female, male, and eggs are shown in Figure 7(A)–(G).

Figure 6. *Lecane bulla.*
Notes: (A) female habitus, (B) male habitus, (C) female with resting egg, (D) unfertilized mictic egg, (E) amictic female egg, (F) amictic and unfertilized mictic eggs.

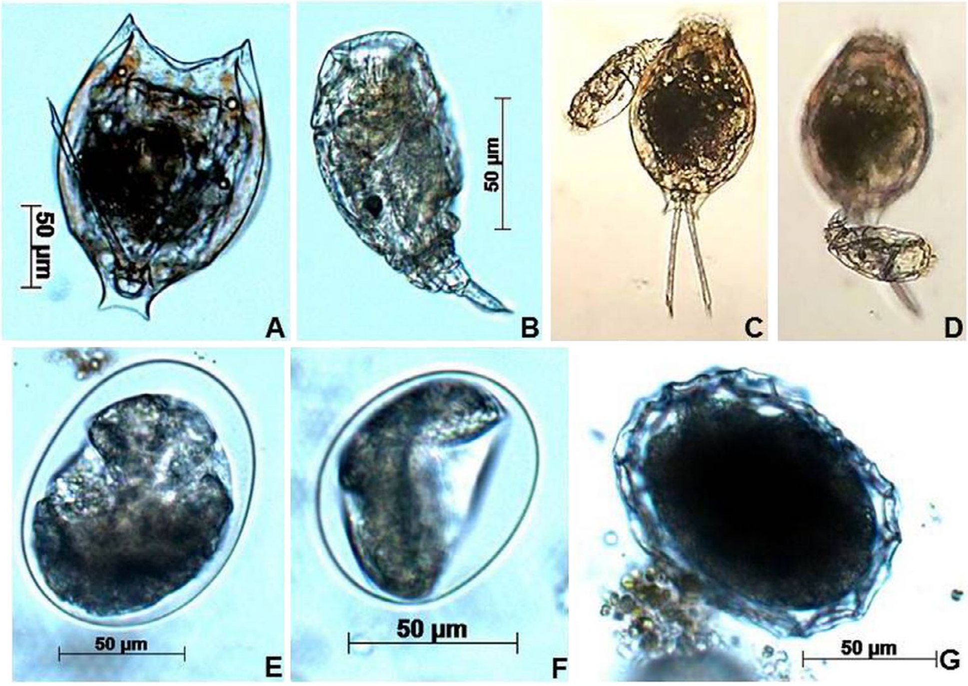

Figure 7. *Lecane leontina*.
Notes: (A) Female in ventral view, (B) male in lateral view, (C) male copulating on the aperture of the corona of female, (D) male copulating with a female at the cloaca, (E) amictic egg, (F) unfertilized mictic egg, (G) resting eggs.

Sexual reproductive behavior of Lecane nana *(Family Lecanidae)*

We did not observe copulation in mating trials of *L. nana*, even after increasing the number of males (10 males per female) or females (three females per male). The density of these cultures was approximately 20 individuals mL^{-1}. However, copulation was observed and resting egg production took place in laboratory cultures with more than 50 individuals ml^{-1}. In supplementary video 8, we observed three males simultaneously contacting a female, and however only one completed the mating behavior sequence. Due to the low quality of supplementary video 8 it is not possible to discern details of the mating behaviors, however we present portions of mating behaviors in supplementary video 8 between the 00:00 to 1:50. Details of the female, male, and eggs of this species are shown in Figure 8(A)–(F).

Sexual reproductive behavior of Trichocerca stylata *(Family Trichocercidae)*

The mating behaviors of *T. stylata* consisted of a fast swimming male that copulated with the female at the foot aperture. The female foot was 41.1 ± 12.8 µm ($n = 21$) and included a substyli or whip that was 319.6 ± 21.4 µm ($n = 21$) when fully extended. This substyli was usually retracted and held near the foot. The substyli can either facilitate or hinder attachment by the male during copulation. The mean time of copulation was 5.1 ± 0.2 m ($n = 2$). However, the female and male remain joined for longer periods of time (even until the death of the male; Figure 8(D)). Morphological details of the female, male, and eggs are shown in Figure 9(A)–(E) and supplementary video 9.

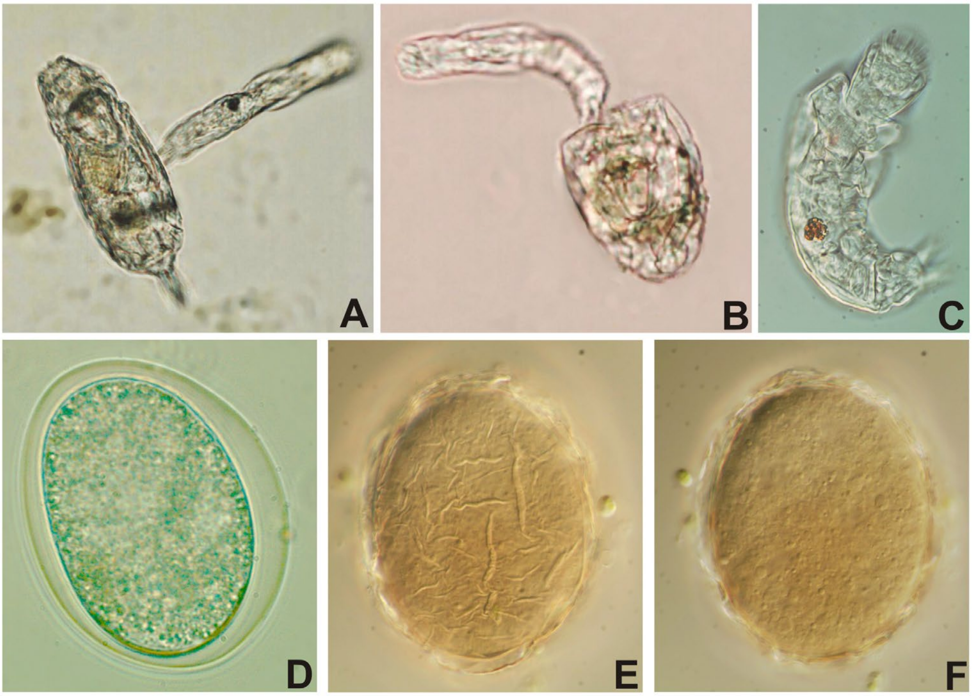

Figure 8. *Lecane nana*.
Notes: (A) male copulating with a female in the mid region of the female body, (B) male copulating with a female at the corona, (C) tubular male, (D) amictic egg; (E) ornamentation of the resting egg, (F) resting eggs.

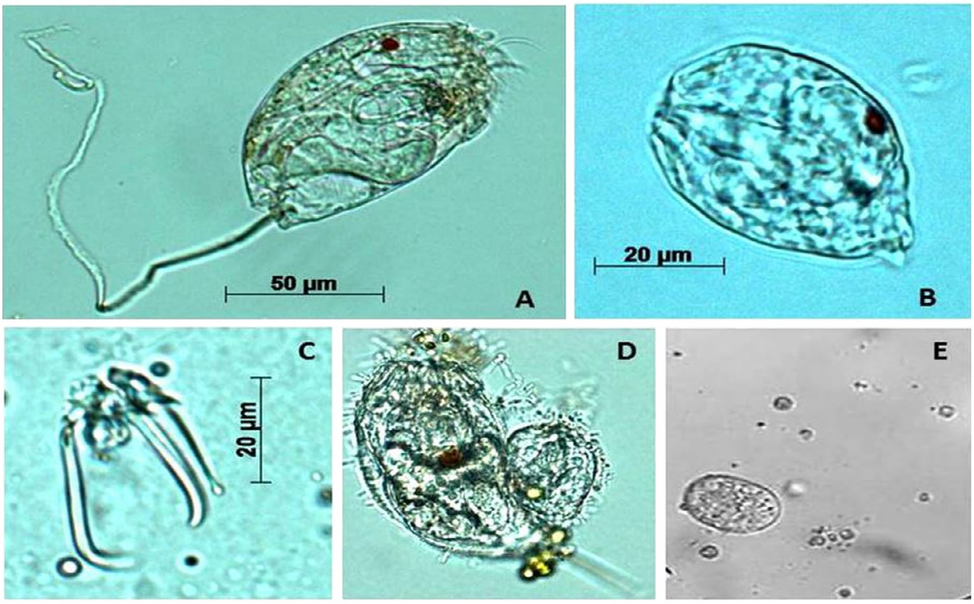

Figure 9. *Trichocerca stylata*.
Notes: (A) female in lateral view, (B) male in lateral view, (C) trophi ventral view, (D) male copulating with a female in the mid region of the body, (E) amictic egg.

Morphological comparison of males of the seven families

Most males of the seven families examined here lack a digestive system and trophi, the exception is some members of the Epiphanidae (e.g. some species of *Rhinoglena*, Ricci & Melone 1998). Detailed description of the males of *Encentrum mucronatum* Wulfert 1936 and *E. martes* Wulfert 1939 (Riemann & Kieneke 2008) were reviewed to aid in the description of males in this study. In general, all males studied here have an unpaired testis with densely packed spermatozoa connected via the vas deferens and contain birefringent bodies. Birefringet bodies may act as energy reservoirs for males (Wallace 1993). Birefringet bodies in males should be separated into those that might be sperm (tightly packed chromosomes are probably birefringent) and energy sources (as in the guts of *Sinantherina socialis* (Linnæus 1758) larvae) (Dr. Robert L. Wallace, pers. comm). Only males of the Brachionidae have tufts of cilia. Only males of Lecanidae have folds in the posterior region near the toes. A comparison of the morphologies of the males representative of the seven families is shown in Figure 10(A)–(F) and selected measurements are given in Table 1. The male to female maximum length ratio ranged from 0.22 to 0.89 among the families studied (Table 1): minimum value *Collotheca ornata* (0.22), maximum value *Cephalodella catellina* (0.89).

Males of *C. catellina* are elongated, but retain an overall conical shape; widest in the frontal trunk region and gradually tapering toward the toes terminating a long pointed foot (Figure 10(A)). In the trunk region, there is a strong dorsal antenna that arises between the cilia of the corona (supplementary videos 3 and 4). The rotatory organ consists of a ciliated field. All cilia are equal of length, but ciliary tufts are absent. The brain is an oval sac that lacks a retrocerebral organ, but lateral antennae are present. The unconspicuous, retractable penis is located in front of toes. As with the other species, the spermatozoa are densely packed (Figure 3(G)).

The male of *B. araceliae* (Brachionidae) was described by Silva-Briano et al. (2007) as having an oval shape. The foot terminates in two toes and a penis with a well-defined corona (Figure 9(B), (C)). All of these characteristics are present in males of other members of the genera *Brachionus*. However, we noted two large ciliary tufts in *B. ibericus* (Figure 10(H)).

The male of *Epiphanes brachionus* (Epiphanidae) has a conical shape, and the rotatory organ consists of a ciliated field, with tufts. The brain is centrally located below the corona. Males have two toes. The swimming pattern is similar to that of other brachionid males (Figures 5(B), 10(G), see supplementary video 2: 2:26 to 3:46 min).

The male of *L. nana* (Lecanidae) is tubular and elongate (Figure 9(C)), and it has the ability to constrict, allowing it to move or slide in on the substratum or to swim (supplementary video 8). The rotatory organ consists of a ciliated field without ciliary tufts. They possess a pair of lateral antenna with few cilia. The body has various folds in the posterior region near the toe (Figure 10(B)). The male of *L. bulla* was first reported by Segers and Rico-Martínez (2000) in Aguascalientes, Central Mexico. The male in this work comes from a population in Cancún, in Southern Mexico. The shapes are similar: head with a conspicuous ciliated corona and a long cylindrical trunk (Figure 10(C)). Toe maximum length is 13.5 ± 8.52 μm ($n = 11$).

The male of *L. leontina* is tubular, contractile, with fold in the posterior region near to the toes, and ovoid in the trunk region. The brain is centrally located (Figure 10(C)). The male is able to constrict, allowing it to move or slide on the substratum or to swim. The

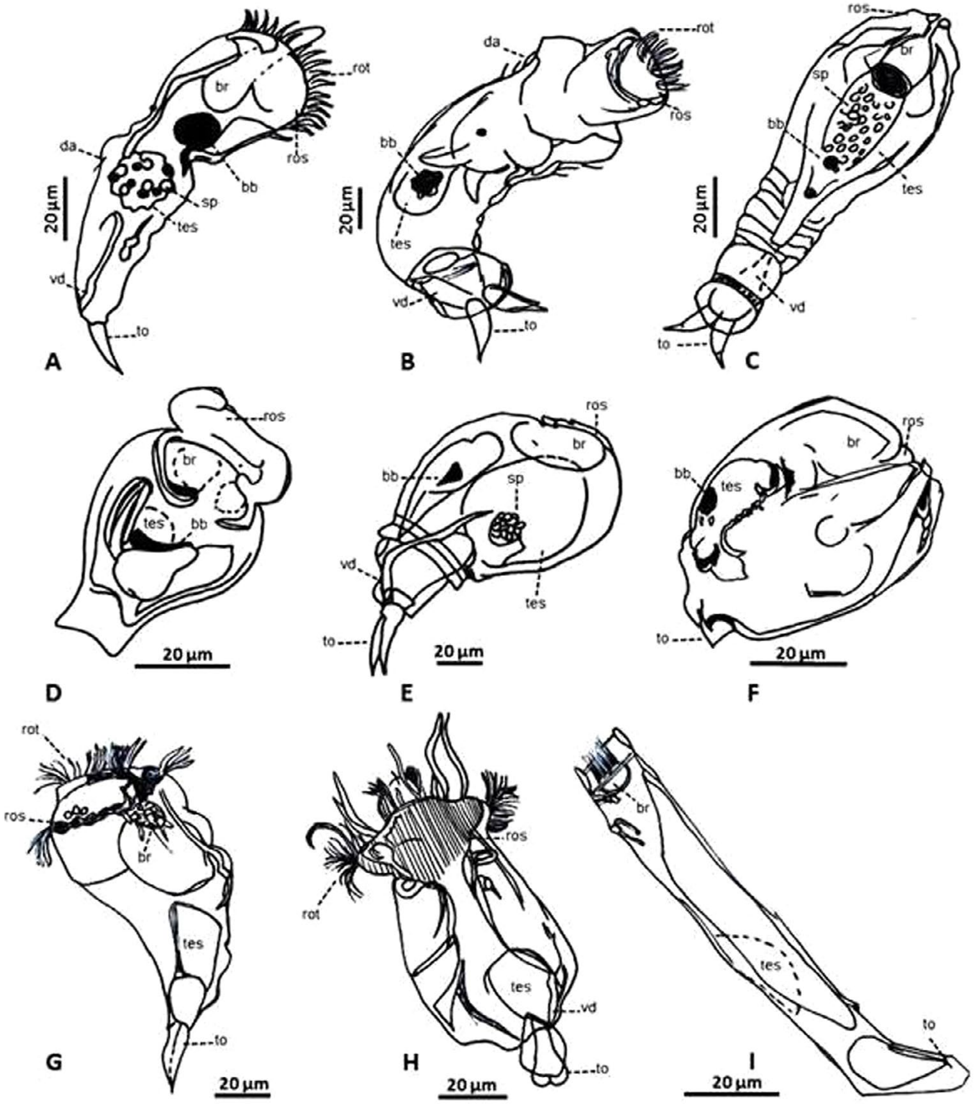

Figure 10. Comparison of males from seven families of rotifers.

Notes: (A) *Cephalodella catellina* (Family Notommatidae); (B) *Lecane nana* (Family Lecanidae), (C) *L. bulla* (Family Lecanidae), (D) *Filinia novazelandiae* (Family Trochosphaeridae) (E) L. leontina (Family Lecanidae), (F) *Trichocerca stylata* (Family Trichocercidae), (G) *Epiphanes brachionus* (Family Epiphanidae), (H) *Brachionus ibericus* (Family Brachionidae), (I) *Collotheca ornata* (Family Collothecidae). Abbreviations: bb = birefringent bodies; br = brain; da = dorsal antenna; ros = rostrum; rot = rotatory organ; sp = spermatozoa; tes = testis; to = toe; vd = vas deferens.

rotatory organ consists of a ciliated field, ciliary tufts are absent. The testis and spermatozoa are present and connected to the vas deferens (Figure 10(E)).

The male of *Filinia novaezealandiae* (Trochosphaeridae) is new to science. It has a conical shape that is slightly wider anteriorly from which the ciliated corona emerges. The posterior part has a squared or blunt end. The rotatory organs have a defined rostrum and a ciliated field. The brain is situated below this structure. The testis is unpaired and contains densely packed spermatozoa. The toes and penis can be difficult to observe; the penis is small and probably contractile (Figure 10(D)). Males have been previously described for: *Trochosphaera aequatorialis* Semper 1872*, T. solstitialis* (Thorpe 1893)*, Filinia passa* (Müller 1786)*, F. longiseta* (Ehrenberg 1834)*,* and *F. cornuta* (Weisse 1847). All *Filinia* males that have been described are similar, including those of *F. novaezealandiae* described here. Interestingly males of the genus *Trochosphaera* differ from those of *Filinia* in that they are globose in the posterior region. Males are unknown in third genera of the family, *Horaella* (Segers 2002).

The male of *T. stylata* (Trichocercidae) (Figure 9(E)) has not been previously described although a size of 60 µm is reported in Koste (1978). This is considerably larger than the size of males measured in this study (43.8 ± 2.3 µm; Table 1). The shape of the male, with an irregular oval body, is similar to that of other species of the genus (Koste 1978). The cephalic ganglion is conspicuous at the dorsal part of body. The maximum length of the foot is 7.0 ± 2.2 µm ($n = 3$), and the penis is inconspicuous.

The male of *C. ornata* (Collothecidae) is planktonic, relatively large, and vermiform (Table 1). In this species, the average male life span was 20 ± 9.4 h ($n = 23$). Male movements included swimming and crawling. The average male movement speed was 0.032 mm/s ± 0.007 ($n = 16$). The rotatory organs have a defined rostrum and a ciliated field. The brain lies below this structure (Figure 10(I)).

Discussion

Prior to this work, knowledge on sexual reproductive behavior of rotifers was limited to seven families (Asplanchnidae, Brachionidae, Epiphanidae, Euchlanidae, Flosculariidae, Lecanidae, Trichocercidae), 11 genera (*Anuraeopsis*, *Asplanchna*, *Brachionus*, *Epiphanes*, *Euchlanis*, *Keratella, Lecane*, *Plationus*, *Platyias*, *Sinantherina*, *Trichocerca*), and 24 species (Gilbert 1963; Rico-Martinez & Snell 1997; Rico-Martínez et al. 1999; Velázquez-Rojas et al. 2002; Schröder & Walsh 2010; Rico-Martinez & Walsh 2013). Here, we provided information for three additional families (Collothecidae, Trochosphaeridae, Notommatidae), three genera (*Cephalodella*, *Collotheca*, and *Filinia*), and nine species. We described sexual reproductive behavior of 11 freshwater and one brackish water (*Brachionus ibericus*) rotifers. Moreover, we described males of five species: *Epiphanes brachionus*, *Filinia novaezealandiae*, *Lecane leontina, Lecane nana,* and *Trichocerca stylata.*

Why do we have information on mating behavior in so few species? The collection of data related to sexual reproductive behavior of rotifers is opportunistic. It is difficult to design experiments and have uniformity of data when in some cases male production lasts only two or three days, often a scant number of males are produced under laboratory conditions, and males are short-lived.

Sexual reproduction in monogonont rotifers is a complex process with major variations among families, genera, and species. For instance, differences in (a) size, shape, and swimming velocity of males, and (b) copulation time and copulation sites among eight species were reported by Rico-Martinez and Snell (1997). Some of these variations were also observed in this study. To date, unique variants in reproductive biology have been reported in five rotifer genera (*Asplanchna*, *Conochilus*, *Ptygura*, *Sinantherina,* and *Synchaeta*). For example, amphoteric females produce both female and male offspring in *Sinantherina socialis* (Rico-Martinez & Walsh 2013) and *Asplanchna* (King & Snell 1977). Another example is the production of pseudosexual eggs produced in the absence of males in some species of *Synchaeta* (Gilbert 1995).

We demonstrate that the typical steps encompassing mating behavior in rotifers (Wallace et al. 2006) are not strictly followed by all species. For instance, circling and coronal localization are either absent or occur infrequently in species of the families: Collothecidae, Lecanidae, Trichocercidae, and Trochosphaeridae (Table 4). Therefore, inclusion of these steps in descriptions of rotifer mating behavior is likely an overgeneralization due to the heavy reliance on brachionid species in studies of mating behaviors and the scarcity of data on a more phylogenetically broad suite of species such as those studied here.

Sexual reproductive behaviors of *Brachionus* conform to a conserved pattern that was also observed in our study of the Mexican endemic species *B. araceliae*. Here we provided evidence of multiple, simultaneous copulations in this species, as well as in *B. quadridentatus*. In addition, we noted that *B. ibericus*, *B. angularis*, and *B. quadridentatus* readily underwent sexual reproduction and produced diapausing embryos for up to two months of laboratory culture. Then, the rate of sexual reproduction decreased and they reproduced primarily asexually.

Table 4. Comparison of mating behavior steps in 12 rotifer species.

Species/mating step	Encounter	Circling	Coronal localization	Sperm transfer	Dissociation
Brachionus angularis	At corona, and foot opening	Always occurs	Always occurs	At corona and foot opening	Present
B. araceliae	At corona	Very short	Always occurs	At corona	Present
B. ibericus	At corona	Always occurs	Always occurs	At corona	Present
B. quadridentatus	At corona	Always occurs	Always occurs	At corona and foot opening	Present
Cephalodella catellina	Near the foot	Absent	Always occurs	Foot opening	Present
Collotheca ornata	At corona	71% of encounters	85% of circling attempts	At the neck region, and coronal lobe	Present
Epiphanes brachionus	At corona and mid-region of lorica	Always occurs	Always occurs	At corona	Present
Filinia novaezealandiae	Near central posterior seta, corona	Always occurs	Occasionally occurs	Near central posterior seta	Present
Lecane bulla	At juncture of ventral and dorsal plates at mid-region of lorica	Absent	Occasionally occurs	At juncture of ventral and dorsal plates at mid-region of lorica	Present
L. nana	Mid-region of lorica, corona	Absent	Occasionally occurs	At juncture of ventral and dorsal plates at mid-region of lorica, and at corona	Present
L. leontina	Near the foot	Absent	Occasionally occurs	Cloaca, and coronal aperture	Present
Trichocerca stylata	Mid-region of lorica, foot	Absent	Absent	Foot opening	Present

Males and copulation

Variation in morphology and copulation strategies in rotifers may be evolutionarily adaptive traits. Gilbert and Williamson (1983) suggested two divergent lines of evolution in male morphology: one favoring fast, small and non-feeding males and the other favoring larger males to avoid cannibalism. Serra and Snell (1998) suggested that dwarf males are a consequence of selection on mictic females to rapidly produce as many males as possible. Ricci and Melone (1998) proposed that selective pressures that affect size and abundance of male rotifers are associated with their habitats. For example, planktonic species need to produce as many male offspring as possible in a short time to locate and mate with females under conditions of high population density. In contrast, sessile rotifers frequently occur under low density conditions and they typically possess larger males that sometimes feed and can survive longer periods of time which would facilitate successful copulation. Thus, the planktonic, small (male to female length ratio ranged from 0.25 to 0.41), non-feeding, short-lived males of the Brachionidae may be a consequence of one set of selective pressures. Whereas it would be predicted that littoral species, such as *Epiphanes brachionus* (Brachionidae), that occur in low densities, would have larger males. In fact the male to female length ratio found for this species was 0.61. In the Lecanidae, species typically inhabit littoral and/or benthic environments, and they tend to have larger males. We found male to female length ratios ranging from 0.55 to 0.88. This is within the range reported for other benthic species, e.g. *Lecane quadridentata* (Ehrenberg 1832): 0.63, and *Euchlanis dilatata* Ehrenberg, 1830: 0.66 (Rico-Martinez & Snell 1997).

In our work examples of other strategies can also be seen. The male of *L. nana*, with its tubular shape, exhibits more contractile and flexible movements. While *C. catellina* males have developed a strong dorsal antenna that can be used to attach to the female during copulation to avoid being dislodged or consumed by the female. The male to female maximum length ratio is slightly greater in *C. catellina* vs. *L. nana* (0.89 vs 0.80).

The sexual reproductive behaviors of *Lecane nana* are different from those reported for *L. quadridentata* (Rico-Martinez & Snell 1997) and *L. bulla* (Segers & Rico-Martínez 2000). The male of *L. nana* is similar to that of *L. psammophila* (Wiszniewski 1932). In contrast, males of other species of this genus (e.g. *L. clara* (Bryce 1892), *L. levystila* (Olofsson 1917), *L. luna* (Müller 1776), *L. quadridentata* (Ehrenberg 1830), and *L. persupilla* (Hauer 1929)) have a conical and elongated shape (Segers 1995), and copulation typically takes place at the junction of the dorsal and ventral plates in a lateral orientation (Rico-Martinez & Snell 1997; Segers & Rico-Martínez 2000).

The males of *Brachionus ibericus*, *B. araceliae*, *B. angularis*, *B. quadridentatus*, and *Epiphanes brachionus* have an ovoid shape and copulation typically takes place at the corona or near the trochal disk of the female. The males of the Trichocercidae described so far are *Trichocerca cylindrica* (Imhof 1891), *T. similis* (Wierzejski 1893), *T. pusilla* (Jennings 1903) by Sudzuki (1958), *T. taurocephala* (Hauer 1931), *T. pediculus* (Remane 1949) by Koste (1978), and *T. porcellus* (Gosse 1851), which has been reported twice (Nipknow 1961; Koste 1978). In our study, we described the male of *T. stylata* for the first time; it is similar in morphology to the male of *T. porcellus*.

Mating behaviors and adaptive strategies of males in sessile and colonial rotifers have been rarely investigated. Previously, *Sinantherina socialis* was the only sessile (and colonial) species with described reproductive biology (Rico-Martinez & Walsh 2013). Here, we described mating behaviors of an additional sessile species, *Collotheca ornata* (Figure 11). The maximum length of *C. ornata* males (100–105 μm) was similar to that reported by Koste (1978). These males are similar to males of other collothecid species such as *C. tenuilobata* (Anderson 1889), *C. campanulata* (Dobie 1849), *C. trilobata* (Collins 1872), *C. pelagica* (Rousselet 1893) (Sarma & Rao 1986), and *Stephanocerus fimbriatus* (Goldfuß 1820) (Wesenberg-Lund 1923). Furthermore, male lifespan (~20 h) of *C. ornata* was almost the same as that of *C. tenuilobata* Anderson 1889 (Sarma & Rao 1986). Whether males mate with free swimming larvae remains to be tested.

In general, the sexual reproductive behaviors, embryonic development, and morphology of males should be studied in yet additional species to allow for better comparisons. For example, in two common rotifer species, *B. calyciflorus* Pallas 1766 and *B. plicatilis* (each from two geographical populations), it was shown that male eggs were deposited much faster than female eggs, but the embryonic development time of these small male eggs was longer than that of larger female eggs (Xu-Wang et al. 2016).

In the analyses of sexual reproduction in these 12 species, we observed differences in copulation time that may be related to sperm transfer. Species of the genus *Brachionus* that copulate quickly (q~1 min) and carry the fertilized eggs outside the female's body generally produced more resting eggs as compared to species of *Cephalodella catellina*, *Lecane bulla*, *Lecane quadridentata* and *Trichocerca pusilla*.

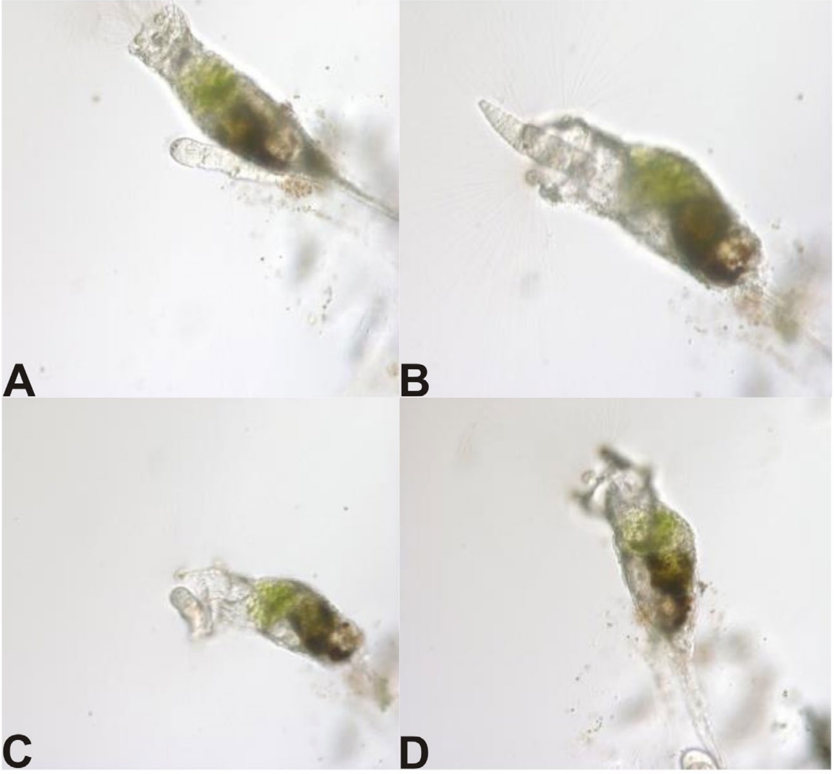

Figure 11. Cannibalism in *Collotheca ornata*.
Notes: (A) female neonate emerging from gelatinous tube; (B–D) adult female ingesting female neonate.

Diapausing embryos (resting eggs)

Diapausing embryos are diploid and possess a thick wall. Females carry the fertilized eggs (or diapausing embryos) in two ways: 1) within the lorica or 2) outside the lorica (Walsh et al. 2017). *Brachionus ibericus* strain Cancún, belonging to the *B. plicatilis* species complex, can be distinguished from other species such as *B. rotundiformis* Tschugunoff 1921, *B. plicatilis* and *B. manjavacas* Fontaneto, Giordani, Melone, and Serra 2007 based on this feature (Mills et al. 2017). In *B. ibericus* the resting egg remains within the lorica and in the other three species the resting eggs are carried outside the lorica (Ciros-Pérez et al. 2001). Some species from other families also vary in this trait. *Lecane quadridentata*, *L. bulla*, and *Filinia novazealandiae* carry resting eggs within the lorica. Species that retain the resting eggs inside the lorica are limited in the number of eggs that they can produce. While the lorica may add an additional layer of protection to the diapausing embryo, it may also be a barrier to the detection of hatching cues. In addition, during predation, eggs carried externally can be released while those carried internally will be consumed. The embryological processes and ecological and evolutionary impacts of how diapausing embryos are carried should be investigated in further detail.

Sexual cannibalism in rotifers

Sexual cannibalism occurs when a female kills and eats a male before or after copulation (Prenter et al. 2006). This phenomenon has not been previously reported in rotifers, although it is well documented in other groups such as spiders (Wilder et al. 2009). Sexual cannibalism in rotifers may represent a survival strategy through optimization of food resources for the female after copulation by providing energy for investment in diapausing embryo production. Because rotifer males are typically much smaller than females, they may be particularly vulnerable to sexual cannibalism. Although, controversial, this behavior can provide added energy for the female, invoke mate choice, and control population size (Prenter et al. 2006). Cannibalism, in general, has been reported in rotifers of the genera *Asplanchna* (Gilbert 1976) and *Cupelopagis* (Bevington et al. 1995), and was observed in *C. ornata* in our study (see Figure 11).

Cephalodella is comprised of 190 species, but ecological, morphometric, and sexual behavior data are quite scarce for this genus (Nogrady & Pourriot 1995). In *C. catellina*, the ability of the male to attach to a female depends on a very robust dorsal antenna (Figure 9). The attachment step is followed by the male bending his body to penetrate the lorica of the female and copulating with the anterior part of the female. Both of these observations are of biological relevance. This behavior may lead to an increase in the probability of successful copulation and at the same time prevent the male from being injured and/or consumed by the female before achieving copulation. Cannibalistic behavior of *C. catellina* must be studied in further detail (as well as in other species) to better understand its biological significance in rotifers. This observation also poses questions such as: (a) How much energy does the female gain by consuming mates? (b) How many males are ingested by an individual female during her lifetime? (c) Is cannibalism limited to the sexual phase of the life cycle? and (d) Do females of other rotifer species cannibalize males?

Sexual reproductive behaviors result from evolutionary pressures that differ among rotifer species. Here we provided some observations of how mating behaviors vary among ecologically and taxonomically diverse species. Our findings suggest that although certain sequences of events must take place (mate recognition, copulation, sperm transfer), there is no conserved pattern of mating behaviors common to all species. Additional differences related to mating include: (a) the shapes and additional structures of males, (b) localization and duration of copulation, (c) optimization of energetic resources through sexual cannibalism, and (d) the position of diapausing embryos (e.g. internally vs. externally). Finally, determination of signals for the induction of sexual reproduction, reproductive efficiency in natural habitats, the documentation of hybridization among species, and the characterization of species complexes are priority lines of research that will aid in our understanding the evolution of sexual reproductive behaviors and genetic recombination in rotifers.

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Disclosure statement

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