ROTIFERA XV

Reproductive isolation, morphological and ecological differentiation among cryptic species of *Euchlanis dilatata*, with the description of four new species

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Abstract Morphological approaches may not provide sufficient resolution for species delineation. Thus, we used an integrated approach that included molecular and ecological characters as well as morphological features to gain a better estimate of species diversity and to improve our understanding of the speciation process within rotifers. Previously, seven putative cryptic species were found within *Euchlanis dilatata* complex based on a nuclear marker. Here, we investigated reproductive isolation, variation in trophi morphology, and life history characteristics among representatives of some of these species. Mating success between each cryptic species was 0–1.1%; lower than that of positive controls (intra-clonal: 15.6–43.9%). SEM trophi images representing individuals from all seven lineages were used for morphometric analyses. Using Discriminant Analysis, 64% of individuals were correctly assigned to cryptic species. Five clonal lineages, each representing a putative species, were used in life table experiments with varying temperature and conductivity. Age-specific fecundity increased under high temperature and life expectancy decreased under high temperature and high conductivity. There was variation among cryptic species in some life table characteristics, such as life expectancy and generation time. Applying an integrative taxonomy approach, we examined the boundaries between *E. dilatata* cryptic species and described four of them as new species.

Keywords Integrative taxonomy · Life table experiments · Speciation · Trophi

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Introduction

Reliable species delineation can be obtained using integrative taxonomy that implements various complementary approaches (Dayrat, 2005); this is especially true for understudied taxa such as microorganisms. Relying solely on morphology to designate species of microorganisms can be misleading and likely does not reflect true levels of species richness and biodiversity as there may not be sufficient recognizable morphological variation among them (Kaya et al., 2009). As a consequence, morphologically similar forms and cryptic species are often lumped together taxonomically (Finlay, 2002).

Rotifers are ubiquitous microorganisms inhabiting various aquatic habitats, making them one of the major constituents of freshwater zooplankton communities (Segers, 2008; Wallace & Snell, 2014). Cryptic diversity can be high in rotifers as they lack easily recognizable morphological characters (Segers, 2008; Fontaneto et al., 2009). Cryptic species in most taxa, including rotifers, are primarily found using molecular analyses (Fontaneto et al., 2015). Most cryptic species within Rotifera have remained at the putative species level and have not been established as new species. Some exceptions include members of the *Epiphanes senta* species complex. *Epiphanes hawaiiensis* Schröder & Walsh, 2007, *E. ukera* Schröder & Walsh, 2007, and *E. chihuahuaensis* Schröder & Walsh, 2007 were described using cross-mating experiments, and morphological and molecular analyses (Schröder & Walsh, 2007). Similarly, *Brachionus manjavacas* Fontaneto, Giordani, Melone & Serra, 2007 (Fontaneto et al., 2007a) and *B. koreanus* Hwang, Dahms, Park & Lee, 2013 (Hwang et al., 2013) in the *B. plicatilis* species complex were described based on molecular and morphological analyses. Integrating molecular and morphological approaches (Papakostas et al., 2016; Michaloudi et al., 2018) resulted in description of *Brachionus elevatus* Michaloudi, Papakostas, Stamou, Neděla, Tihlaříková, Zhang, Declerck, 2018 and *B. fernandoi* Michaloudi, Papakostas, Stamou, Neděla, Tihlaříková, Zhang, Declerck, 2018.

Although DNA taxonomy is not always sufficient to define species boundaries, it can be considered an effective tool to complement morphology-based taxonomy (Packer et al., 2009). DNA taxonomy provides hypotheses about species that can be tested with morphological, behavioral, ecological, and mate choice studies, and to eventually describe cryptic species. Using several lines of evidence to describe cryptic species aids in substantiating the taxonomic status of new species.

Genetic studies are only capable of indicating whether or not cryptic species have exchanged genetic materials. However, it is not possible to determine whether they have lost the ability for such exchange (Bickford et al., 2007). Mate choice experiments can examine the strength of reproductive isolation among cryptic species (e.g., in rotifers: Gómez et al., 1995; Gilbert & Walsh, 2005; Schröder & Walsh, 2007, 2010; Wiwegweaw et al., 2009). Although mating experiments have the potential to confirm

whether genetic variation among cryptic species of monogonont rotifers is accompanied by reproductive isolation (Rico-Martinez & Snell, 1995; Schröder & Walsh, 2007), few studies have focused on examining this among rotifer cryptic species. For example, Xiang et al. (2011) carried out cross-mating experiments among cryptic species of *Brachionus calyciflorus* Pallas, 1766 and considered the production of resting eggs as the indicator of successful mating. Others have studied pre- and post-mating reproductive isolation among cryptic species (e.g., *B. plicatilis* species complex: Gómez et al., 1995; Rico-Martinez & Snell, 1995; Gómez & Snell, 1996; Suatoni et al., 2006; *B. calyciflorus* species complex: Gilbert & Walsh, 2005; *E. senta* species complex: Schröder & Walsh, 2007, 2010). Examining reproductive isolation and its drivers can increase confidence in delimiting species boundaries and furthermore describing them as new species.

Studying morphological variation is another approach that can complement results obtained through DNA taxonomy. For rotifers, morphological variation among cryptic species has been found in some complexes. For example in the *B. plicatilis* complex, variation in lorica size was used to differentiate between *B. plicatilis* and *B. rotundiformis* Tschugunoff, 1921 (Fu et al., 1991). Similarly, lorica size and spine patterns vary between *B. rotundiformis* and *B. ibericus* Ciros-Pérez, Gómez & Serra, 2001 (Ciros-Pérez et al., 2001). Hwang et al. (2013) showed that *B. koreanus* has a smaller lorica with shorter distances between pairs of inner and outer anterior spines as compared to *B. plicatilis*. In the *E. senta* complex, morphological variation in trophi and resting egg features, along with DNA evidence and cross-mating experiments, led to the designation of new species (Schröder & Walsh, 2007, 2010). In bdelloid rotifers, Fontaneto et al. (2007b) found variation in trophi size and shape within seven bdelloid morphospecies. However in other studies, no robust morphological variation was found among cryptic species. For instance, Fontaneto et al. (2007a) noted there are no robust morphological differences between *B. plicatilis* and *B. manjavacas* based on geometric morphometric analyses of lorica and trophi. Leasi et al. (2013) did not find any significant variation in morphological features of lorica and body size among cryptic species of *Testudinella clypeata* (Müller, 1786). In a study by Malekzadeh-Viayeh et al. (2014), there was no

significant variation in lorica morphology between *B. plicatilis* 'Tiscar' and a putative new lineage from Iran. Although morphological analyses may not always be sufficient to represent all of the diversity that is identified through molecular methods. However in some cases, they can be applied to distinguish among and to help describe some cryptic species of rotifers.

Ecological niche differentiation can promote reproductive isolation and consequently speciation (Schluter, 2001). By incorporating physical and chemical features of the habitat—in the study of cryptic species, it is possible to examine whether occurrence of a cryptic species is correlated to specific environmental factors (e.g., Ortells et al., 2003; Dennis & Hellberg, 2010; Obertegger et al., 2012; Papakostas et al., 2013; Zhang et al., 2015), and beyond that, whether ecological differentiation is a driver of speciation (e.g., Liu et al., 2013; Fouet et al., 2017). Moreover, investigating ecological adaptation among cryptic species can test the species boundaries specified by molecular methods (Rissler & Apodaca, 2007). Temperature is an environmental factor that affects age-specific survivorship and reproduction rate of zooplankton, including rotifers (e.g., Galkovskaja, 1987; Ma et al., 2010; Johnston & Snell, 2016). Furthermore, variation in response to temperature has been documented among some cryptic species from a variety of protists and rotifers (e.g., 15 protist species: Rose et al., 2008; dinoflagellate: *Akashiwo sanguinea* species complex: Luo et al., 2017; rotifers: *B. ibericus*, *B. plicatilis,* and *B. rotundiformis*: Gómez et al., 1995; *B. calyciflorus*: Li et al., 2010). While the relationship between conductivity and zooplankton species composition and abundance has also been well studied (e.g., Bos et al., 1996; Soto & Rios, 2006; Çelik & Ongun, 2007; Sousa et al., 2008; Walsh et al., 2008; Fatema et al., 2016; Celewicz-Goldyn & Kuczynska-Kippen, 2017), few studies have addressed the differential response to conductivity among cryptic species. Pfenninger & Nowak (2008) showed that two cryptic species of the midge *Chironomus piger* Strenzke, 1956 can tolerate higher levels of conductivity compared to *C. riparius* (Meigen, 1804). Obertegger et al. (2014) found that the occurrence of cryptic species in the rotifer *Polyarthra dolichoptera* Bartoš, 1951 in high-altitude lakes was related to conductivity as well as to longitude, and silica concentrations. Differential response of cryptic species to conductivity levels can contribute to ecological niche differentiation, thus providing information on ecological variation among them.

Despite the observed reproductive isolation and variation in ecological adaptation among cryptic species of rotifers, many of them are not described. In a few studies where cryptic species were described (see above), morphological variation has been found among them. Cryptic species are likely to be left unnamed if they are not morphologically distinguishable. This cryptic diversity can be described based on their diagnostic nucleotides. This method has been successfully used to describe cryptic species of other taxa (e.g., skipper butterfly *Astraptes fulgerator* (Walch, 1775), Brower, 2010; sea snail *Alviniconcha hessleri* Okutani & Ohta, 1988, Johnson et al., 2015; amphipod *Niphargus stygius* (Schiödte, 1847), Delic et al. 2017) and has been recommended for this purpose by Jörger & Schrödl (2013).

In this study, we investigated reproductive isolation and variation in trophi morphology among members of the *Euchlanis dilatata* Ehrenberg, species complex. In addition, we conducted life table experiments to examine variation in survivorship, fecundity, generation time, net reproductive rate, and intrinsic rate of population increase among five cryptic species of the *E. dilatata* complex in response to water temperature and conductivity. We used diagnostic nucleotides in the ITS region to describe four *E. dilatata* cryptic species. Finally, a neotype for the species was designated.

Materials and methods

In a study by Kordbacheh et al. (2017), ITS region and COI gene sequences from 62 populations of the *Euchlanis dilatata* species complex were analyzed to measure genetic differentiation and identify cryptic species. Molecular species delimitation was based on ITS region sequences and at least seven putative cryptic species were found. Clonal isolates derived from populations representing selected cryptic species (previously included in the molecular analysis) were used in the following experiments. Populations were selected based on availability of laboratory cultures. Additional details are available in Supplemental Document S1 (Figure S1.1, Table S1.2) and Kordbacheh et al. (2017).

Cross-mating experiments

Clonal lineages from 11 populations of the *Euchlanis dilatata* complex were used in mating experiments within and between six putative cryptic species (Table 1). Each clonal lineage was initiated using one asexual female. Clonal lineages were cultured in modified MBL media (Stemberger, 1981) at room temperature and were fed a mixture of the algae *Rhodomonas minuta* Skutja, 1948, *Chlorella vulgaris* Berijerinck, 1890 (Culture Collection of Algae at the University of Texas at Austin [UTEX] strain 30) and *Chlamydomonas reinhardtii* Dangeard, 1888 (UTEX strain 90).

To conduct mating experiments, female embryos and males were isolated from cultures that were mictic under laboratory conditions. We did not control for male age, however slow swimming males (indicative of old age) were not used. Two female embryos and two males were used in each mating trial. Females and males obtained from the same clonal lineage (intra-clonal) were included as positive controls. For cross-mating experiments (inter-clonal), females and males were obtained from different lineages. Trials were checked after 48 h and the success of mating (e.g., the production of resting eggs) was recorded. If the female embryos did not hatch or the neonate produced amictic eggs, the trial was excluded from statistical analyses. Cross-mating experiments were reciprocal, for example between males from lineage 'A' and females from lineage 'B' and vice versa. We conducted six combinations of mating experiments between putative cryptic species that were either sister groups (e.g., species 'A' and 'B', see Supplemental Document S1, Figure S1.1) or had low genetic distance based on the ITS region sequences ($\leq 3.2\%$ e.g., species 'C' and

Table 1 Collection site of *Euchlanis dilatata* clonal lineages used in mating experiments, and the lineage and species that they were assigned

Collection site	Collection date	GPS coordinates (decimal degrees)	ITS region lineage delimitation	Species
Rio Grande, Williamsburg, Sierra Co., NM (Williamsburg)	07.24.2013	33.110039/− 107.297839	'A'	*E. dilatata* s.s.
Mescalero Lake, Otero Co., NM (Mescalero)	07.05.2013	33.2984/− 105.6886	'A'	*E. dilatata* s.s.
Fish Pond, Nockamixon State Park, Bucks Co., PA (NockFish)	05.20.2016	40.472567/− 75.224823	'B'	*E. kingi* sp. nov.
Feather Lake Wildlife Sanctuary, El Paso Co., TX (Feather Lake)	10.10.2007	31.6890972/− 106.3052666	'C'	*E. texana* sp. nov.
Rio Grande, American Dam, El Paso Co., TX (American Dam)	12.19.2013	31.784234/− 106.527845	'C'	*E. texana* sp. nov.
Former cattle tank sediment, White Sands National Monument, Doña Ana Co., NM (Cattle Tank)	09.20.2006	32.67485/− 106.44345	'D'	*E. chihuahuaensis* sp. nov.
Red Tank, Hudspeth Co., TX (Red Tank)	06.04.2014	30.7303083/− 104.9891083	'D'	*E. chihuahuaensis* sp. nov.
Cinnamon Bay Beach pond, St. John, U.S. Virgin Islands (CBB)	05.24.2012	18.356281/− 64.752136	'E'	Not described
Cattail Falls, pool C', Brewster Co., TX (Cattail C')	08.08.2006	29.2731833/− 103.3361638	'F'	*E. floridensis* sp. nov.
Cattail Falls, pool G, Brewster Co., TX (Cattail G)	06.23.2013	29.2731666/− 103.3361638	'F'	*E. floridensis* sp. nov.
Krome pond, Miami-Dade Co., FL (Krome)	11.24.2015	25.883615/− 80.484920	'F'	*E. floridensis* sp. nov.

Modified from Kordbacheh et al. (2017). The reference system for coordinates is the World Geodetic System 1984 (WGS84). Abbreviations are shown in parentheses and are used in Tables 2 and 3

'D', Supplemental Document S1, Table S1.2). The genetic distance in ITS region sequences was substantial between cryptic species 'F' and the other five cryptic species, ranging from 9.7 to 10.3%. We crossed this species with species 'E' (genetic distance: 10.3%) and species 'D' (genetic distance: 9.7%). Species 'E' was crossed with species 'C' and 'D' as well. We also used four inter-clonal combinations of mating experiments within cryptic species. Moreover, 11 intra-clonal combinations were used as positive controls (Tables 2, 3). Success of each mating experiment was determined by dividing the number of successful trials (e.g., fertilized resting egg produced) by the total number of trials. Inter-clonal mating success was compared to success found in positive controls. To test whether there was a significant difference in mating success between inter-clonal and intra-clonal trials, Pearson's χ^2 test was implemented in RStudio v 1.1.383 (RStudio Team, 2016). Clonal lineages were considered to be reproductively isolated when inter-clonal mating success rate was significantly lower than intra-clonal mating success rate.

Trophi morphology

Trophi, frontal and caudal view, for populations representing all seven putative cryptic species from the *E. dilatata* complex were prepared for scanning electron microscopy (SEM) by dissolving rotifer tissue in $\sim$ 5% sodium hypochlorite, extracting the trophi and rinsing them with deionized water approximately 15 times, and air-drying them on circular cover slips at room temperature (modified from Segers, 1993). Trophi were coated with gold/palladium using a Gatan 682 PECS sputter coater. A Hitachi S-4800 scanning electron microscope located in UTEP's Metallurgical, Materials and Biomedical Engineering department was used to obtain SEM images at 20 kV.

Images of trophi were observed and examined for features to distinguish among putative cryptic species. Length of rami, manubria, and fulcrum were measured for 175 trophi representing seven putative cryptic species ('A' (*n* = 32), 'B' (*n* = 4), 'C' (*n* = 44), 'D' (*n* = 23), 'E' (*n* = 32), 'F' (*n* = 36), and 'G' (*n* = 4)) using a Zeiss Axioscope equipped with a SPOT camera and Basic Software v 5.2 (Diagnostic Instruments, Inc). Discriminant Analysis in SPSS v 24.0 (IBM Corp, 2016) was used to determine the percentage of individuals that were correctly assigned to cryptic species based on the size of measured trophi elements.

Life table experiments

Five clonal lineages representing five putative cryptic species from the *E. dilatata* complex collected from the Rio Grande at Williamsburg, Sierra Co., NM (cryptic species 'A'), the Rio Grande at American Dam, El Paso Co., TX (cryptic species 'C'), a former cattle tank, White Sands National Monument, Doña Ana Co., NM (cryptic species 'D'), a small temporary pond at Cinnamon Bay Beach, St. John, U.S. Virgin Islands (cryptic species 'E'), and a pond alongside Krome Ave., Miami-Dade Co., FL (cryptic species 'F') were selected for life table experiments. Populations from cryptic species 'B' and 'G' were not available in the laboratory at the time of the experiments. Age-specific survivorship (l_x), fecundity (m_x), generation time (*T*), net reproductive rate (R_0), and the intrinsic rate of population increase (*r*) were examined under four conditions: (1) Low temperature, low conductivity (tc: 20°C, conductivity at 180 μS/cm), (2) Low temperature, high conductivity (tC: 20°C, conductivity at 1800 μS/cm), (3) High temperature, low conductivity (Tc: 27°C, conductivity at 180 μS/cm), and (4) High temperature, high conductivity (TC: 27°C, conductivity at 1800 μS/cm). The high values for water temperature and conductivity in these treatments were greater than what was recorded in the field for most studied populations. In field collections where members of the *E. dilatata* species complex were found, the highest recorded conductivity was in the Rio Grande at Williamsburg, NM ($\sim$ 1,600 uS/cm); this population was also found at the highest water temperature, 27.7°C. The conductivity of modified MBL medium was 180 μS/cm. To raise the conductivity to 1800 μS/cm, 0.73 g NaCl was added to 1 L of MBL medium. Each treatment initially consisted of four replicates, each with nine females. Female neonates (< 6 h old) were placed into nine well culture plates with 0.8 ml of modified MBL medium containing the alga *C. reinhardtii* (500,000 ± 50,000 cell/ml) and were incubated using a 18:6 h light:dark photoperiod. Females were checked every 12 h to determine survival and the number of eggs and offspring that were produced until the death of the last individual. Offspring were

removed from the culture plates as they hatched. Females were moved to new food-containing medium every 24 h. Mictic females, females lost during the experiment, or those that died in the first 24 h were excluded from the analyses, as were replicates with fewer than five individuals. Therefore in the final analysis, the number of females in each replicate ranged from 5 to 9. The following formulae were used to calculate age-specific survivorship (l_x), fecundity (m_x), net reproductive rate (R_0), generation time (T), and intrinsic rate of population increase (r):

$$l_x = s_x/s_0$$

$$m_x = b_x/n_x$$

$$R_0 = \sum_{x=0}^{k} lxmx$$

$$T = \sum_{x=0}^{k} \frac{xlxmx}{R_0}$$

$$r = \ln(R_0)/T$$

where x is time (day), n_x is the number of females, s_x is the number of survivors, and b_x is the number of offspring produced each day.

We used a Linear Mixed-Effects Model in RStudio v 1.1.383 (RStudio Team, 2016) with fixed effects of temperature, conductivity, cryptic species, and their interactions to determine differences in l_xm_x and e_x; variation among individuals was considered as a random effect. To determine significant differences in generation time (T), net reproductive rate (R_0), and intrinsic rate of population increase (r) among cryptic lineages we used the exact permutation test in R v 3.4.3 (R core team, 2017).

Species accounts

We described cryptic species 'B', 'C', 'D', and 'F' from the E. dilatata complex (Kordbacheh et al., 2017) following Jörger & Schrödl (2013). In the study by Kordbacheh et al. (2017) mitonuclear discordance was found, thus species delimitation was based on the ITS marker as it yielded more conservative results as compared to COI gene sequences. Moreover, ITS region sequences have been found to be a more reliable marker for species delimitation in the B.

plicatilis and B. calyciflorus species complexes (Mills et al., 2017 and Papakostas et al., 2016, respectively). Based on these findings, in this study the position and character state of diagnostic nucleotides corresponding to the alignment of ITS sequences in MAFFT v 7 (Katoh & Standley, 2013) were used for species diagnosis (849 bp including insertions; Supplemental Document S2).

Because there is no record of a type specimen for Euchlanis dilatata corresponding to its original description of (Ehrenberg, 1830), a specimen from the most widespread species in the complex (species 'A') was selected as a neotype. Following the recommendation of Jörger and Schrödl's (2013), species 'E' and 'G' were not described because they were each represented by only one population in the phylogenetic analysis by Kordbacheh et al. (2017).

Results

Cross-mating experiments

The intra-clonal mating success rate (positive controls) ranged from 15.6 to 43.9%. Within cryptic species mating success rate was 1.2 to 17.9% and between cryptic species success rate was 0 to 1.1% (Table 2). Within cryptic species, the probability of inter-clonal mating success was lower than intra-clonal mating success (χ^2 value = 0.04–47.4, $P < 0.001$). The exception was crosses between Cattail Falls pool C' and G from cryptic species 'F'. In this case mating success was not different from that of positive controls ($\chi^2 = 0.035$, df = 1, $P = 0.8$). Further, resting eggs were produced in all mating experiments within cryptic species (Table 2). However, it should be noted that the viability of embryos was not determined.

For inter-clonal crosses between cryptic species, the success rate was 0 for all crosses except for the crosses between individuals from Cattail Falls pool C', TX (cryptic species 'F') and Cinnamon Bay Beach pond, USVI (cryptic species 'E'). In this cross, one resting egg was produced out of 40 trials between males from cryptic species 'F' and females from cryptic species 'E'. However, no resting eggs were produced in 49 trials when females from cryptic species 'F' were crossed with males from cryptic species 'E'. The genetic variation between these

cryptic species was 10.3% in the ITS region and 13.5% in the COI gene sequences (Kordbacheh et al., 2017).

Based on χ^2 test results, the probability of interbreeding between cryptic species was significantly lower than the probability of inbreeding within clonal lineages (Tables 2, 3).

Trophi morphology

Discriminant Analysis showed that only 64% of individuals were assigned correctly to their cryptic species based on trophi measurements (Wilks' Lambda = 0.08, χ^2 = 78, $P < 0.001$, Fig. 1). Species 'A' was the most distinguishable, with 83% of individuals assigned correctly. Moreover, all trophi analyzed from individuals representing four populations of species 'A' had a small projection on the left ramus, which was not observed in the other putative cryptic species (Fig. 2). In Discriminant Analysis, Function 1 (71.3% of variation) was associated with fulcrum length and Function 2 (14.8% of variation) was associated with manubria length showing most variation in trophi size was related to fulcrum and manubria length.
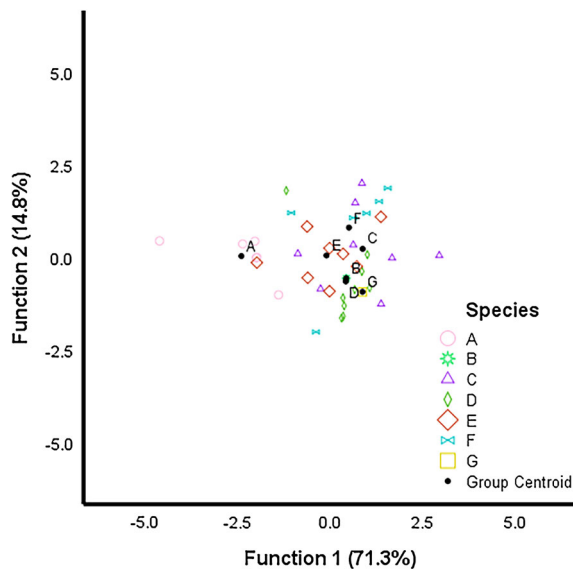
Fig. 1 Discriminant Analysis based on length of rami, manubria, and fulcrum of trophi from seven *Euchlanis dilatata* cryptic species. The amount of variation explained by the first two discriminant functions is shown in parentheses

Life table experiments

Age-specific survivorship (l_x), fecundity (m_x), and life expectancy (e_x)

Lineage, water temperature, and the interaction between temperature and lineage and the interaction between temperature, lineage and conductivity had significant effects on $l_x m_x$ (Linear Mixed-Effects Model, $P < 0.001$). Lineage ($P < 0.01$), water temperature ($P < 0.01$), conductivity ($P = 0.01$), and the interaction between temperature and lineage ($P = 0.01$) had significant effects on e_x (for more details, see Supplemental Document S3, Table S3.1). Higher values of $l_x m_x$ were observed at temperature 27°C (Fig. 3, Table 4). Life expectancy decreased under high temperature and high conductivity (Table 4).

Only significant differences among cryptic species in each treatment are reported below. In all cases, the P value was 0.03, unless otherwise specified (Supplemental Document S3, Table S3.2).

Generation time (T)

For all cryptic species, generation time (T) was the longest under tc conditions. Cryptic species 'A' and 'E' had the shortest generation time under TC treatments while cryptic species 'C', 'D', and 'F' had the shortest generation time under Tc treatments (Table 4).

In tc treatments, generation time ranged from 4.8 to 5.6 days, but there was no significant difference among cryptic species. Under tC, generation time ranged from 3.3 to 5.4 days. In these treatments, cryptic species 'E' had the longest generation time (mean ± standard error: 5.4 ± 0.06) and was significantly different from cryptic species 'A' (3.9 ± 0.75) and 'D' (4.6 ± 0.17). Under Tc, generation time ranged from 2.2 to 3.2 days. Cryptic species 'A' (3.1 ± 0.18) and 'E' (3.2 ± 0.17) had longer generation time compared to cryptic species 'C' (2.2 ± 0.09) and 'F' (2.7 ± 0.15). Under TC, generation time ranged from 2.7 to 3.3 days. In these treatments, generation time of cryptic species 'E' (3.1 ± 0.06) and 'C' (3.3 ± 0.22, $P = 0.05$) was longer than that of cryptic species 'D' (2.7 ± 0.09) (Table 4 and Supplemental Document S3, Table S3.2).
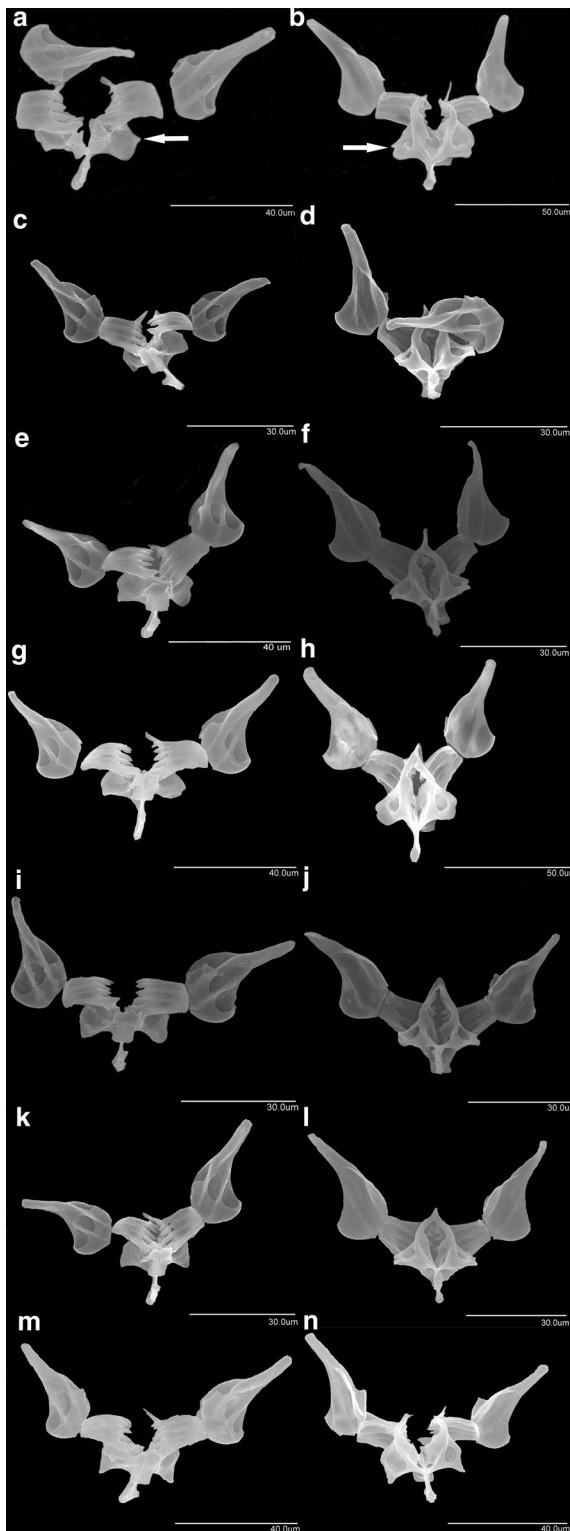
◄**Fig. 2** Trophi of the *Euchlanis dilatata* **a** cryptic species 'A' frontal view, **b** cryptic species 'A' caudal view, **c** cryptic species 'B' frontal view, **d** cryptic species 'B' caudal view, **e** cryptic species 'C' frontal view, **f** cryptic species 'C' caudal view, **g** cryptic species 'D' frontal view, **h** cryptic species 'D' caudal view, **i** cryptic species 'E' frontal view, **j** cryptic species 'E' caudal view, **k** cryptic species 'F' frontal view, **l** cryptic species 'F' caudal view, **m** cryptic species 'G' frontal view, **n** cryptic species 'G' caudal view. Arrows indicate the projection on left ramus of cryptic species 'A' trophi

Net reproductive rate (R_0)

Cryptic species 'C', 'D', and 'F' showed the highest net reproductive rate (R_0) under TC. Cryptic species 'A' had the highest R_0 under Tc. Cryptic species 'E' had the highest R_0 under tc. For cryptic species 'A' and 'F', the lowest R_0 was under tC conditions. Cryptic species 'D' and 'E' had their lowest R_0 under Tc. Cryptic species 'C' showed the lowest value of R_0 under tc and tC (Table 4 and Supplemental Document S3, Table S3.2).

Under tc, R_0 ranged from 1.0 to 9.2. Cryptic species 'F' showed the lowest R_0 (1.0 ± 0.17) that was significantly lower than the other four cryptic species. Net reproductive rate of cryptic species 'D' (9.2 ± 1.54) was significantly higher than that of 'A' and 'C'. Cryptic species 'C' (3.7 ± 0.39) had a lower R_0 compared to cryptic species 'E' (6.3 ± 0.68). Under tC, R_0 ranged from 0.5 to 10.9. Net reproductive rate of cryptic species 'D' (10.9 ± 0.96) was the highest, and that of cryptic species 'F' (0.5 ± 0.15) was the lowest in these treatments. Species 'A' had a higher R_0 than that of species 'F' ($P = 0.05$). Under Tc, R_0 ranged from 0.7 to 9.4. Net reproductive rate of cryptic species 'A' (9.4 ± 1.04) was the highest and R_0 of cryptic species 'F' (0.7 ± 0.25) was the lowest in these treatments. Net reproductive rate of cryptic species 'C' (5.2 ± 0.73) was lower than that of cryptic species 'A' ($P = 0.05$). Cryptic species 'D' (3.1 ± 0.60) had lower R_0 than cryptic species 'C'. Under TC, R_0 ranged from 1.2 to 11.6. Net reproductive rates of cryptic species 'E' (1.2 ± 0.43) and 'F' (1.3 ± 0.40) were significantly lower compared to the other three cryptic species (Table 4 and Supplemental Document S3, Table S3.2).

Fig. 3 Age-specific fecundity ($l_x m_x$) of five *Euchlanis dilatata* cryptic species under four conditions: (1) Low temperature, low conductivity (tc: temperature: 20°C, conductivity: 180 µS/cm), (2) Low temperature, high conductivity (tC: temperature: 20°C, conductivity: 1800 µS/cm), (3) High temperature, low conductivity (Tc: temperature: 27°C, conductivity: 180 µS/cm), and (4) High temperature, high conductivity (TC: temperature: 27°C, conductivity: 1800 µS/cm). Each line on the graph represents a cryptic lineage
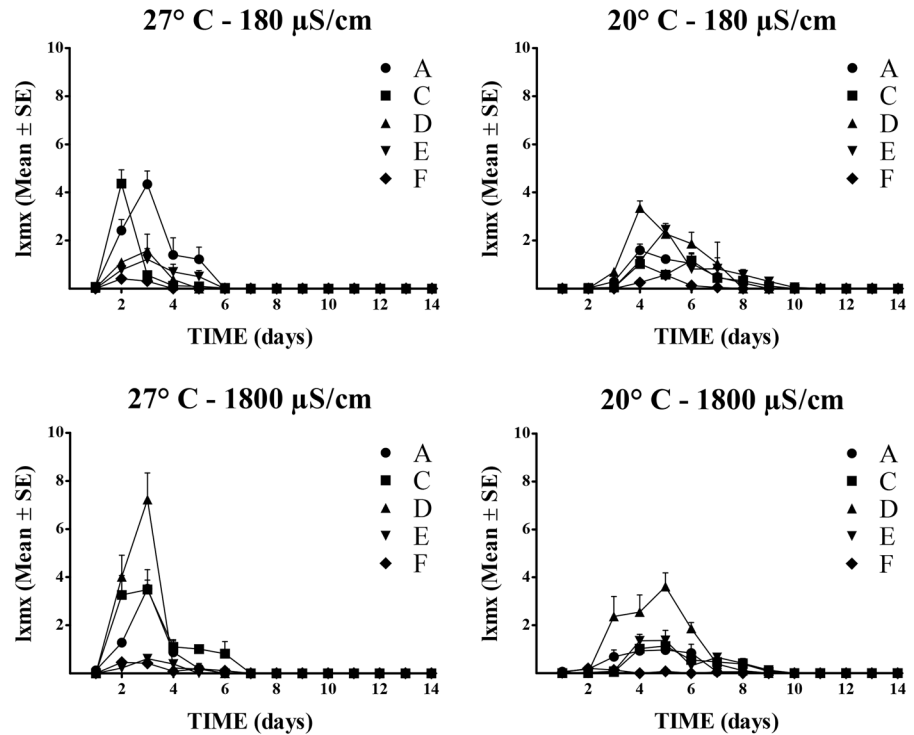
Intrinsic rate of population increase (r)

Cryptic species 'A' had the highest intrinsic rate of population increase (r) under Tc treatments. Cryptic species 'C' showed the highest r under Tc and TC. Cryptic species 'E' had the highest value of r under tc and Tc. Cryptic species 'D' and 'F' had the highest r under TC conditions. Intrinsic rate of population increase was the lowest under tC conditions for cryptic species 'A'. Cryptic species 'F' had the lowest values of r under tC and Tc. Cryptic species 'C' showed the lowest value of r under tc and tC conditions. For cryptic species 'D', the lowest value of intrinsic rate of population increase was under tc treatments. Cryptic species 'E' had the lowest r under TC (Table 4 and Supplemental Document S3, Table S3.2).

Under tc, r was − 0.02 to 0.4. Cryptic species 'D' (0.4 ± 0.02) had the highest and cryptic species 'F' (− 0.02 ± 0.04) showed the lowest values of r in these treatments. The r of cryptic species 'A' (0.3 ± 0.01) and 'E' (0.3 ± 0.01) were larger than cryptic species 'C' (0.2 ± 0.01). Under tC, r increase ranged from − 0.3 to 0.5. The r of cryptic species 'D' (0.5 ± 0.03) was higher than the other cryptic species

(between cryptic species 'A' and D, $P = 0.05$). Cryptic species 'F' (− 0.3 ± 0.15) had a lower r than cryptic species 'A' (0.2 ± 0.11) ($P = 0.05$), 'C' (0.2 ± 0.03), and 'E' (0.2 ± 0.04). Under Tc, r was − 0.3 to 0.7. The r of cryptic species 'A' (0.7 ± 0.05) and 'C' (0.7 ± 0.04) were higher than the other three cryptic species. Cryptic species 'D' (0.4 ± 0.08) showed a higher r than 'F' (− 0.3 ± 0.22). Under TC, r ranged from − 0.04 to 0.90. The r of cryptic species 'E' (− 0.04 ± 0.17) and 'F' (0.01 ± 0.09) were lower than that of cryptic species 'A' (0.6 ± 0.02), 'C' (0.7 ± 0.08), and 'D' (0.9 ± 0.05) (between cryptic species 'A' and 'E', $P = 0.05$). Cryptic species 'A' had a lower r than cryptic species 'D' ($P = 0.05$) (Table 4; Supplemental Document S3, Table S3.2). Negative value of r indicates population size was decreasing.

Discussion

Euchlanis dilatata is a species complex with at least seven cryptic species (Kordbacheh et al., 2017). In this study, we observed reproductive isolation among six

Table 2 Overall intra- and inter- clonal mating success rate (%) for populations of *Euchlanis dilatata*

Population	Population	Mating success rate (%)	Trials (n)	Trial level
Williamsburg ('A')	Williamsburg ('A')	32.5	40	IC
Mescalero ('A')	Mescalero ('A')	42.5	40	IC
NockFish ('B')	NockFish ('B')	41.5	41	IC
Feather Lake ('C')	Feather Lake ('C')	23.6	93	IC
American Dam ('C')	American Dam ('C')	27.4	51	IC
Red Tank ('D')	Red Tank ('D')	42.8	70	IC
Cattle Tank ('D')	Cattle Tank ('D')	43.9	41	IC
CBB ('E')	CBB ('E')	36.5	123	IC
Cattail C' ('F')	Cattail C' ('F')	15.6	115	IC
Cattail G ('F')	Cattail G ('F')	22.4	67	IC
Krome ('F')	Krome ('F')	22.2	36	IC
Williamsburg ('A')	Mescalero ('A')	1.25	80	ICWS
Feather Lake ('C')	American Dam ('C')	6.7	89	ICWS
Red Tank ('D')	Cattle Tank ('D')	3.2	92	ICWS
Cattail C' ('F')	Cattail G ('F')	17.9	39	ICWS
Williamsburg ('A')	NockFish ('B')	0	92	ICBS
Feather Lake ('C')	Red Tank ('D')	0	83	ICBS
Feather Lake ('C')	CBB ('E')	0	84	ICBS
Red Tank ('D')	CBB ('E')	0	85	ICBS
Red Tank ('D')	Cattail G ('F')	0	72	ICBS
CBB ('E')	Cattail C' ('F')	1.1	89	**ICBS**

Cryptic species that clonal lineages belong are shown in parentheses. Mating experiments included reciprocal trials. IC: intra-clonal trial, ICWS: inter-clonal within cryptic species trial, ICBS: inter-clonal between cryptic species trial. Population name abbreviations are defined in Table 1

Table 3 χ^2 tests used to examine whether intra-clonal mating success was significantly higher than inter-clonal mating success for both within and between cryptic species trials of *Euchlanis dilatata*

Clonal lineages	Cryptic species	Number of trials	χ^2 value	P value
Williamsburg versus NockFish	'A' versus 'B'	92	47.5	< 0.001
Feather Lake versus Red Tank	'C' versus 'D'	83	52.2	< 0.001
CBB versus Feather Lake	'E' versus 'C'	84	41.5	< 0.001
CBB versus Red Tank	'E' versus 'D'	85	52.2	< 0.001
Cattail C' versus CBB	'F' versus 'E'	89	15.3	< 0.001
Cattail G versus Red Tank	'F' versus 'D'	72	22.5	< 0.001
Williamsburg versus Mescalero	'A' versus 'A'	80	47.4	< 0.001
American Dam versus Feather Lake	'C' versus 'C'	89	12.8	< 0.001
Red Tank versus Cattle Tank	'D' versus 'D'	92	34.1	< 0.001
Cattail C' versus Cattail G	'F' versus 'F'	39	0.04	0.8

Mating experiments included reciprocal trials. Abbreviations are defined in Table 1

and ecological differentiation among five of them. Moreover, one of the cryptic species (cryptic species 'A') showed morphological differences in trophi shape and size from other cryptic species within the complex.

Reproductive isolation can be mediated through pre-mating and/or post-mating reproductive barriers. Reproductive isolation has been examined among cryptic species of many aquatic invertebrates [e.g.,

Lee (2000); Addison & Kim (2018)]. In rotifers, pre-mating barriers include differentiation in timing and cues of shifting from asexual to sexual reproduction (e.g., *B. plicatilis*: Carmona et al., 1995; *E. hawaiiensis* and *E. chihuahuaensis*: Schröder & Walsh, 2010), and failure to recognize individuals of other species as potential mates (e.g., *B. plicatilis* species complex: Kotani et al., 1997; Gribble & Mark Welch, 2012). Post-mating barriers include the lack of resting egg

Table 4 Generation time (T), net reproductive rate (R_0), intrinsic rate of population increase (r), age-specific fecundity ($l_x m_x$), and life expectancy (e_x) for five cryptic species of *Euchlanis dilatata* [mean ± standard error]

Cryptic species	Parameter	tc	tC	Tc	TC
'A'	n	34	31	31	18
	T	5.1 ± 0.30	3.9 ± 0.75	3.1 ± 0.18	2.9 ± 0.18
	R_0	5.0 ± 0.34	3.8 ± 1.53	9.4 ± 1.04	6.0 ± 0.92
	r	0.3 ± 0.01	0.2 ± 0.11	0.7 ± 0.05	0.6 ± 0.02
	$l_x m_x$	0.6 ± 0.18	0.5 ± 0.25	1.9 ± 0.45	1.2 ± 0.27
	e_x	2.1 ± 0.41	1.7 ± 0.41	1.6 ± 0.18	1.1 ± 0.18
'C'	n	36	36	31	32
	T	5.6 ± 0.14	5.4 ± 0.23	2.2 ± 0.09	3.3 ± 0.22
	R_0	3.7 ± 0.39	3.7 ± 0.78	5.2 ± 0.73	9.7 ± 2.08
	r	0.2 ± 0.01	0.2 ± 0.03	0.7 ± 0.04	0.7 ± 0.08
	$l_x m_x$	0.4 ± 0.09	0.4 ± 0.12	0.9 ± 0.16	1.6 ± 0.43
	e_x	2.3 ± 0.22	2.3 ± 0.48	1.3 ± 0.22	1.5 ± 0.27
'D'	n	33	33	34	34
	T	4.8 ± 0.15	4.6 ± 0.17	2.7 ± 0.19	2.7 ± 0.09
	R_0	9.2 ± 1.54	10.9 ± 0.96	3.1 ± 0.60	11.6 ± 1.78
	r	0.4 ± 0.02	0.5 ± 0.03	0.4 ± 0.08	0.9 ± 0.05
	$l_x m_x$	1.3 ± 0.31	1.4 ± 0.34	0.8 ± 0.29	2.3 ± 0.48
	e_x	2.3 ± 0.14	2.1 ± 0.15	1.0 ± 0.13	1.0 ± 0.10
'E'	n	35	33	18	36
	T	5.6 ± 0.23	5.4 ± 0.06	3.2 ± 0.17	3.1 ± 0.06
	R_0	6.3 ± 0.68	4.4 ± 0.80	3.2 ± 0.61	1.2 ± 0.43
	r	0.3 ± 0.01	0.2 ± 0.04	0.3 ± 0.05	- 0.04 ± 0.17
	$l_x m_x$	0.6 ± 0.16	0.5 ± 0.13	0.6 ± 0.22	0.3 ± 0.12
	e_x	2.4 ± 0.36	2.3 ± 0.21	1.5 ± 0.18	1.0 ± 0.13
'F'	n	34	33	24	35
	T	4.8 ± 0.29	3.3 ± 0.88	2.7 ± 0.15	2.9 ± 0.34
	R_0	1.0 ± 0.17	0.5 ± 0.15	0.7 ± 0.25	1.3 ± 0.40
	r	− 0.02 ± 0.04	− 0.3 ± 0.15	− 0.3 ± 0.22	0.01 ± 0.09
	$l_x m_x$	0.1 ± 0.07	0.1 ± 0.06	0.2 ± 0.08	0.2 ± 0.09
	e_x	1.4 ± 0.37	1.4 ± 0.15	0.8 ± 0.07	1.1 ± 0.17

tc 20°C, 180 µS/cm; *tC* 20°C, 1800 µS/cm; *Tc* 27°C, 180 µS/cm; *TC* 27°C, 1800 µS/cm; *n* indicates the number of individuals per treatment

production after copulation, production of unviable resting eggs, and/or female mortality after copulation (e.g., *Asplanchna brightwelli* Gosse, 1850: King, 1977; *E. ukera* and *E. chihuahuaensis*: Schröder & Walsh, 2007) and unviable and/or sterile F1 females (e.g., *B. plicatilis* species complex: Suatoni et al., 2006). In this study, resting eggs were not produced in cross-mating trials between cryptic species, with one exception. A single resting egg was produced in a mating between a female of cryptic species 'E' and a male of cryptic species 'F', despite high genetic differentiation between them based on both markers. We did not determine whether this resting egg was viable. Therefore, we cannot be certain whether gene flow between these two species occurs. On the other hand, cryptic species 'A' and 'B' were reproductively isolated based on our results. The genetic distance between cryptic species 'A' and 'B' in the ITS region was 1.2% and 11.1% in the COI gene. It is possible that when there is no or limited gene flow between cryptic species of the *E. dilatata* complex, the COI gene accumulates genetic variation at a higher rate compared to the ITS region due to faster rate of evolution (Cruickshank, 2002; Vilas et al., 2005). Overall, the inter-clonal mating success rates within cryptic species were lower than the positive controls, and all the within cryptic species cross-mating experiments were successful. Thus, suggesting gene flow

occurs within cryptic species although at a lower rate than between species.

True cryptic species are not easily distinguishable based on morphology (Bickford et al., 2007). However, morphological variation among cryptic species of rotifers has been reported (see Fu et al., 1991; Ciros-Pérez et al., 2001; Campillo et al., 2005; Anitha & George, 2006; Schröder & Walsh, 2007; Fontaneto et al., 2007b; Hwang et al. 2013; Michaloudi et al., 2016). Here, we examined trophi morphology of representatives of populations from the *E. dilatata*. Based on trophi size, in general only 64% of individuals were assigned correctly to cryptic species. Individuals of cryptic species 'A' had the highest rate of accurate assignment to their cryptic species (83%). In addition to variation in trophi size for cryptic species 'A', we observed a projection on the left ramus of trophi that was specific to this cryptic species. A population of the *E. dilatata* species complex collected in Italy had a similar projection (Parise, 1966). Molecular analyses would be necessary to determine whether that population is assigned to species 'A'. Based on our results, at least six cryptic species of the *E. dilatata* complex are indistinguishable based on trophi morphology. Lack of variation in trophi among these cryptic species may represent morphological stasis despite high genetic variation. Yet, differentiation in body size related to ploidy level has been reported within *E. dilatata* populations (Walsh & Zhang, 1992). Life history characteristics under high and low temperatures also varied with ploidy level (Walsh, 1992). It is possible that body size as well as other morphological features vary among cryptic species.

We observed variation in life table parameters among five examined cryptic species that may stem from genetic variation among them as noted for net reproductive rate of *B. calyciflorus* cryptic species (Wang et al., 2014) and some life history features such as age-specific fecundity for lineages of *Keratella cochlearis* (Gosse, 1851) (Cieplinski et al., 2018). For example in our study, cryptic species 'D' had the highest age-specific fecundity ($l_x m_x$) under all experiment conditions except Tc, and species 'F' had the lowest values of $l_x m_x$ and net reproductive rate under all experiment conditions. This variation indicates that *E. dilatata* lineages are evolving independently and can be treated as species.

Life history parameters of rotifers can be affected by many factors (Table 5). In this study, temperature had a significant effect on age–specific fecundity ($l_x m_x$), and life expectancy (e_x), while conductivity only significantly impacted e_x. As poikilotherms, rotifers are known to exhibit a positive correlation between development rates and temperature, while life expectancy tends to be negatively correlated with temperature (e.g., Miracle & Serra, 1989; Pavón-Meza et al., 2005; Ogello et al., 2016; Saucedo-Ríos et al., 2017). Pavón-Meza et al. (2005) recorded lower life expectancy and shorter generation time, and higher net reproductive rate and population growth at a temperature 25°C as compared to 15°C for *B. havanaensis* Rousselet, 1911. Similarly, Ogello et al. (2016) observed longer life expectancy at a lower temperature for a Kenyan strain of the rotifer *Brachionus angularis* Gosse, 1851. In our study, all five cryptic species from the *E. dilatata* complex tested had higher age-specific fecundity and lower life expectancy at temperature 27°C as compared to 20°C.

Generation time decreases under high temperatures for many poikilotherms (Gillooly, 2000) including rotifers (e.g., Galkovskaja, 1987; Pavón-Meza et al., 2005; Xiang et al., 2010; Wang et al., 2014). We observed the longest generation time for all cryptic species under tc. With an increase of temperature, the generation time for all species decreased. Increase of temperature affects the rate of metabolic activities including developmental processes and thus results in increased r (Miracle & Serra, 1989). We also noted all cryptic species had higher R_0 and r at 27°C. For cryptic species 'D' and 'F', the highest R_0 and r were under TC. Cryptic species 'C' had its highest R_0 under TC and the highest r under Tc and TC. In this study, under high temperatures, cryptic species showed variation in generation time, reproduction rate, and population growth in response to water conductivity. Because we used NaCl to increase the conductivity of MBL to 1,800 μS/cm^2, conductivity in our experiments represents salinity. In a review by Miracle and Serra (1989), it was noted that for *Brachionus dimidiatus* Bryce, 1931 and *B. plicatilis*, r decreases as the salinity concentration deviates from the optimal level. Differential response to water salinity among cryptic species of rotifers has been addressed. *Brachionus plicatilis* had higher reproduction rate compared to *B. manjavacas* in lower salinities (Gabaldón et al., 2015). On the other hand, Leasi et al. (2013) did not find variation

Table 5 Example of studies on life history characteristics of selected rotifers under a variety of conditions

Species	Examined variable(s)	Reference(s)
Asplanchna brightwellii	Predation	Santos Medrano et al. (2016)
Brachionus calyciflorus	Water temperature	Xiang et al. (2010) and Wang et al. (2014)
	Salinity	Wang et al. (2014)
	Food type and/or food density	Kirk (1997), Li et al. (2010) and Pan et al. (2016)
	Hatching from diapausing versus subitaneous eggs	Gilbert (2004)
	Heavy metal concentration	Ramírez-Pérez et al. (2004) and Pan et al. (2016)
B. manjavacas	Water temperature and salinity	Gabaldón et al. (2015)
	Caloric restriction	Gribble & Mark Welch (2012)
B. plicatilis	Water temperature and salinity	Gabaldón et al. (2015)
	Salinity, food type and/or food density	Malekzadeh-Viayeh et al. (2010) and Gorokhova (2017)
B. urceolaris	Salinity, food type and/or food density	Malekzadeh-Viayeh et al. (2010)
Euchlanis dilatata	Food type and/or food density	Espinosa-Rodríguez et al. (2012) and Farhadian et al. (2013)
	Competition	Espinosa-Rodríguez et al. (2012)
	Methyl parathion concentration	Sarma et al. (2001)
Plationus patulus	Heavy metal concentration	Ríos-Arana et al. (2007)
Keratella spp.	Predation	Neil (1984)
Synchaeta pectinata	Water temperature	Stelzer (2002)
	Food type and/or food density, caloric restriction	Kirk (1997)

in the occurrence of cryptic species of the *Testudinella clypeata* species complex in relation to salinity.

Life history characteristics of *E. dilatata* has been studied, for instance in response to competition and/or food type (Espinosa-Rodríguez et al., 2012; Farhadian et al., 2013), combined effects of methyl parathion and food density (Sarma et al., 2001), cadmium, lead, mercury, and methyl parathion concentration (Arias-Almeida & Rico-Martínez, 2011), and predation (Nandini et al., 2011). Moreover, Walsh & Zhang (1992) found that a large morphotype of *E. dilatata* was triploid and had a longer time to maturity as compared to that of a smaller diploid morphotype. Here, we provided evidence of variation in survivorship, reproduction rate, and population growth in response to water temperature and conductivity among genetically distinct isolates. Kordbacheh et al. (2017) reported co-occurrence of cryptic species 'C' and 'D' in Laguna Prieta, El Paso Co., TX. Cryptic species 'C' was hatched from rehydrated sediments and an active population representing cryptic species

'D' was collected in 2013. These two species may be temporarily isolated, that could have resulted from the ecological differentiation between them. Cryptic species of *B. plicatilis* that show ecological variation have been found in sympatry by Montero-Pau et al. (2011) and Wang et al. (2014). Ecological niche differentiation has been recorded between *E. dilatata* and *E. dilatata lucksiana* (Hauer, 1930) where the latter, even in sympatry with *E. dilatata*, has been found only from planktonic habitats (Adamkiewicz-Chojnacka, 1988). It should be noted that *E. dilatata* cryptic species were collected from habitats with different pHs, temperatures, and conductivities. For example, species 'C' and 'D' were collected from habitats with water temperatures > 30°C and they showed the highest R_0 under high temperature and high conductivity conditions. Species 'F' was found in habitats with pH and conductivity as low as 5.9 and 405 μS/cm, respectively. However, it did not show a significant difference in life history characteristics between high and low conductivity conditions.

Species 'F' may be the most ecologically differentiated lineage because it had the lowest $l_x m_x$ and R_0 under all experiment conditions. The observed variation in life history parameters highlights potential ecological niche differentiation among *E. dilatata* cryptic species, and thus it may be a major contributor to the genetic divergence among cryptic species.

Cryptic species of several invertebrates have been described based on diagnostic nucleotides (e.g., Brower, 2010; Johnson et al., 2015; Delic et al. 2017). Although we did not find morphological variation in trophi features among cryptic species of the *E. dilatata* complex (except for cryptic species 'A'), we documented reproductive isolation among six, and ecological differentiation among five cryptic species. We described four cryptic species from the *E. dilatata* complex using diagnostic nucleotides in the ITS region sequence following Jörger & Schrödl's (2013) guidelines as well as designated a neotype. In a study with description of two new species within *B. calyciflorus* complex, Michaloudi et al. (2018) selected the lineage with highest variation in the morphology of the antero-dorsal spines as the neotype. Here, we designated the cryptic species (i.e., lineage 'A') with widest geographic distribution and that has a feature of the rami that was illustrated in a drawing of *E. dilatata* trophi based on a European population (Parise, 1966) as the neotype.

To better understand mechanisms involved in speciation within the *E. dilatata* complex and other rotifer species complexes, future studies should focus on identifying pre- and post-mating reproductive barriers among cryptic species within this species complex such as variation in ploidy level, mixis induction, differentiation in mating behaviors, and the viability of hybrids.

To further study the ecological niche differentiation, other environmental variables such as food density, food type, and competition should be included in life table experiments among cryptic species, and the genetic basis of variation in ecological adaptation should be explored. Biological and ecological species concepts can be used to delimit species within rotifers and other microorganisms by investigating reproductive isolation and ecological differentiation among them.

Species accounts

Euchlanis dilatata was first described by Ehrenberg (1830). However, there is no known type specimen (Segers et al., 2012) and the type locality was not specified (Ehrenberg, 1830). Therefore, following Article 75.3.4 of the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature, 1999), we designated a specimen from cryptic species 'A' and from the population collected from the Rio Grande, Sierra Co., NM, USA as a neotype for *Euchlanis dilatata* sensu stricto. A representative from this lineage was selected because it had the widest geographic distribution compared to other cryptic species analyzed this study. Moreover, the small projection on the ramus observed in this species was also depicted in a drawing of a specimen from a population collected in Italy (Parise, 1966).

Nomenclatural considerations: Because no type specimen is known for *E. dilatata* and we are describing new species within this species complex, we designate a neotype to stabilize the taxonomic identity of the name. The neotype is deposited in a public repository, the National Museum of Natural History, Smithsonian Institution (USNM), for future studies.

Euchlanis dilatata sensu stricto

Neotype locality: Rio Grande, Sierra Co., NM, USA, 33.110039, − 107.297839, 1288 m.

Diagnosis: This species is distinguished from other cryptic species of *Euchlanis dilatata* complex in this study based on the following character states: 156A; 350T (MAFFT alignment of ITS region sequences; Supplemental Document S2). The morphological diagnostic feature is a small projection on the left ramus (Fig. 2).

Neotype: The neotype is an amictic female on a permanent microscopic slide, deposited in the National Museum of Natural History, Smithsonian Institution (USNM 1532147). Additional materials consist of one permanent microscopic slide containing 3 amictic females and 30 amictic females preserved in 95% ethanol deposited in both the National Museum of Natural History, Smithsonian Institution (USNM 1532148 and USNM 1532149, respectively) and UTEP Biodiversity Collections (UTEP:Zoo:69), and the trophi specimen deposited in the UTEP

Biodiversity Collections (UTEP:Zoo:69). All deposited specimens are from the same clonal lineage. ITS region and COI gene sequences are deposited in GenBank (accession numbers, ITS: KU665929-45, COI: KU665853-69).

Other examined populations: Detroit Lake State Park, Marion Co., OR, USA (44.700829, − 122.185241), Centinela Creek, Imperial Co., CA, USA (33.987892, − 118.406403), Chicken Creek Reservoir, Juab Co., UT, USA (39.49185, − 111.95760), Santaquin Reservoir, Juab Co., UT, USA (39.955778, − 111.780283), Alto Reservoir, Lincoln Co., NM, USA (33.395833, − 105.671944), Bear Canyon Lake, Grant Co., NM, USA (32.8846, − 107.998033), Mescalero Lake, Otero Co., NM, USA (33.296660, − 105.687849), Storrie Lake, San Miguel Co., NM, USA (35.6606694, − 105.2345833), Rio Grande, Fabens, El Paso Co., TX, USA (31.430121, − 106.142488), Walter E. Long Lake, Travis Co., TX, USA (30.284417, − 97.608383), Shafter stream, Presidio Co., TX, USA (29.8143166, − 104.3071333), Lake Travis, Travis Co., TX, USA (30.402417, − 97.947967), Oknoname 085006 Reservoir, Love Co., OK, USA (33.927583, − 97.301067), Lake Okeechobee, Glades Co., FL, USA (26.830456, − 80.941303), Lake Mendota, Dane Co., WI, USA (43.079504, − 89.419193), Madeleine Lake, Oneida Co., WI, USA (45.889733, − 89.643833), Schmitthenner Lake, Wyoming Co., PA, USA (41.43738, − 76.24304), Panther Hollow Lake, Allegheny Co., PA, USA (40.436856, − 79.948825), Silver Creek, Schuylkill Co., PA, USA (40.543206, − 75.304106), Presa de la Boquilla, Chihuahua, Mexico (27.5361333, − 105.4011333), Lago Colina, Chihuahua, Mexico (27.5724, − 105.4004666), Presa Chihuahua, Chihuahua, Mexico (28.5762166, − 106.1711833), Poza Azul, Coahuila, Mexico (26.926417, − 102.122538).

Morphology: Body is oval. Lorica is truncated at the anterior and rounded at the posterior part (Fig. 4). Cross-section of the lorica is arc of circle. Foot is slender with two joints, toes are parallel-sided with sharp tip. Trophi has four stout unci teeth, with inner denticulate comb at the tip of rami. Fulcrum is club shaped. There is a small projection on the left ramus (Fig. 2). Dorsal plate length 132 to 270 μm.

Ecology and distribution: In our study, this species was mostly found in streams, permanent lakes and reservoirs from Texas, Wisconsin, Oregon, California, Pennsylvania, New Mexico, Florida, and Mexico. pH ranged from 7.7 to 9.0, temperature ranged from 14.7 to 27.7°C and conductivity ranged from 230 to 1638 μS/cm.

New species descriptions

Euchlanis kingi sp. nov.

urn:lsid:zoobank.org:act:6E65C5BB-2046-4498-BE6D-891CD2F620B7

Type locality: Fish Pond, Nockamixon State Park, Bucks Co., PA, USA, 40.472567, − 75.224823, 131 m.

Diagnosis: This species is distinguished from other cryptic species of *Euchlanis dilatata* complex in this study based on following character states in the ITS region: 309C; a deletion at 350.

Holotype: An amictic female from cryptic species 'B' on a permanent microscopic slide is deposited in the National Museum of Natural History, Smithsonian Institution (USNM 1532150). Paratypes (one permanent microscopic slide of amictic females, and approximately 30 amictic females preserved in 95% ethanol) are deposited in the National Museum of Natural History, Smithsonian Institution (USNM 1532151 and USNM 1532152, respectively) and UTEP Biodiversity Collections (UTEP:Zoo:91). ITS region and COI gene sequences are deposited in GenBank (accession numbers, ITS: KX714930, COI: KX714920).

Etymology: This species is named after Charles E. King because he was the first to hypothesize that genetically distinct lineages of *Euchlanis dilatata* are temporally isolated because of differentiation in ecological adaptation.

Other examined populations: American River, San Joaquin Co., CA, USA (38.57221207, − 121.3531190), Echo Lake, El Dorado Co., CA, USA (38.834557, − 120.046143), Lodi Lake, San Joaquin Co., CA, USA (38.147222, − 121.292723), Timber Lake ditch, Mount Hood National Forest, Clackamas Co., OR, USA (45.083424, − 122.050234).

Morphology: Body is oval. Lorica is truncated at the anterior and rounded at the posterior part (Fig. 4). Cross-section of the lorica is arc of circle. Foot is slender with two joints, toes are parallel-sided with
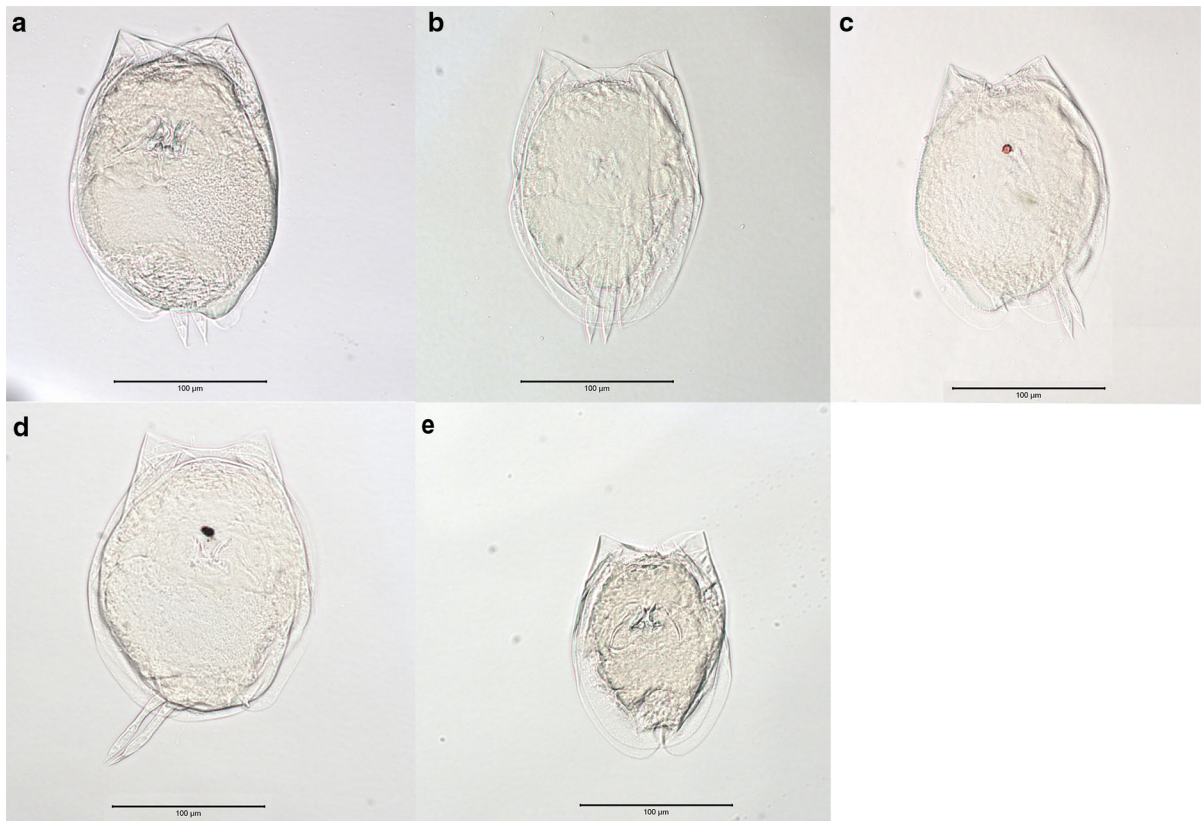
Fig. 4 Amictic females representing five species within the *Euchlanis dilatata* complex. **a** *Euchlanis dilatata* s.s., **b** *E. kingi* sp. nov., **c** *E. texana* sp. nov., **d** *E. chihuahuaensis* sp. nov., and **e** *E. floridensis* sp. nov

sharp tip. Trophi has four stout unci teeth, with inner denticulate comb at the tip of rami. Fulcrum is club shaped (Fig. 2). Dorsal plate length 140 to 200 μm.

Ecology and distribution: In our study, this species was found in ponds, lakes and streams from California, Pennsylvania, and Oregon. For one population that was collected from a drainage ditch near Timber Lake, OR: pH was 6.7, temperature was 17.1°C and conductivity was 104 μS/cm. Environmental information was not available for other populations representing this species.

Euchlanis texana sp. nov.

urn:lsid:zoobank.org:act:14D2DCB3-8037-4049-B6BB-DD69B9C02649

Type locality: Rio Grande, El Paso Co., TX, USA, 31.784234,

− 106.527845, 1136 m.

Diagnosis: This species is distinguished from other cryptic species of *Euchlanis dilatata* complex in this study based on following character states in the ITS region: 644C.

Holotype: An amictic female from cryptic species 'C' on a permanent microscopic slide is deposited in the National Museum of Natural History, Smithsonian Institution (USNM 1532153). Paratypes (one permanent microscopic slide of amictic females, and approximately 30 amictic females preserved in 95% ethanol) are deposited in the National Museum of Natural History, Smithsonian Institution (USNM 1532154 and USNM 1532155, respectively) and UTEP Biodiversity Collections (UTEP:Zoo:127). ITS region and COI gene sequences are deposited in GenBank (accession numbers, ITS: KU665946, COI: KU665851).

Etymology: The species name refers to the state of Texas because most populations representing this species are found in Texas, USA.

Other examined populations: Triangle Pond, Buenos Aires National Wildlife Refuge, Pima Co., AZ, USA (31.55, − 111.533889), Behind Mescalero Canyon playa, Hueco Tanks State Park and Historic Site, El Paso Co., TX, USA (31.9190694, − 106.0408305), Corral Tank, Hudspeth Co., TX, USA (30.8237166, − 105.3155833), Feather Lake Wildlife Sanctuary, El Paso Co., TX, USA (31.6890972, − 106.3052666), Glenn Springs, pool 3, Big Bend National Park, Brewster Co., TX, USA (29.1744166, − 103.1575), Glenn Springs, pool 6, Big Bend National Park, Brewster Co., TX, USA (29.1744166, − 103.1575), Roadside Pond, Jefferson Co., TX, USA (29.834722, − 94.482222), Laguna Prieta, Hueco Tanks State Park and Historic Site, El Paso Co., TX, USA (31.9246388, − 106.046675), Mesocosm, Hueco Tanks State Park and Historic Site, El Paso Co., TX, USA (31.9188166, − 106.040366), Miller Ranch, Jeff Davis Co., TX, USA (30.623845, − 104.674005), Sam Rayburn State Park, Sabine Co., TX, USA (31.061244, − 94.106127), Palo Pinto Canyon stream, Presidio Co., TX, USA (30.0308666, − 104.4684333), Lake Okeechobee, Glades Co., FL, USA (26.830456, − 80.941303), Shorty Howell pond, Gwinnett Co., GA, USA (33.97455, − 84.14936), La Mesa Canyon Tule spring, Lower Canyons, Mexico (29.75111, − 102.58305).

Morphology: Body is oval. Lorica is truncated at the anterior and rounded at the posterior part (Fig. 4). Cross-section of the lorica is arc of circle. Foot is slender with two joints, toes are parallel-sided with sharp tip. Trophi has four stout unci teeth, with inner denticulate comb at the tip of rami (Fig. 2). Fulcrum is club shaped. Dorsal plate length 120 to 184 μm.

Ecology and distribution: In our study, this species was found in a variety of habitats from temporary ponds and playas to permanent lakes from Texas, Florida, Arizona and Mexico. pH ranged from 6.4 to 9.4, temperature ranged from 7.0 to 33.6°C and conductivity ranged from 179 to 1110 μS/cm.

Euchlanis chihuahuaensis sp. nov.

urn:lsid:zoobank.org:act:DDE39A67-7024-4FDA-B915-C2E76F52E043

Type locality: Former cattle tank, White Sands National Monument, Doña Ana Co., NM, USA, 32.67485, − 106.44345, 1213 m.

Diagnosis: This species is distinguished from other cryptic species of *Euchlanis dilatata* complex in this study based on following character states in the ITS region: 366A; 756T.

Holotype: An amictic female from cryptic species 'D' on a permanent microscopic slide is deposited in the National Museum of Natural History, Smithsonian Institution (USNM 1532159). Paratypes (one permanent microscopic slide of amictic females, and approximately 30 amictic females preserved in 95% ethanol) are deposited in the National Museum of Natural History, Smithsonian Institution (USNM 1532160 and USNM 1532161, respectively) and UTEP Biodiversity Collections (UTEP:Zoo:24). ITS region and COI gene sequences are deposited in GenBank (accession numbers, ITS: KU665978, COI: KU665883).

Etymology: The species name refers to Chihuahuan Desert in North America from where most populations of this species were collected.

Other examined populations: Mescalero Canyon playa, Hueco Tanks State Park and Historic Site, El Paso Co., TX, USA (31.9188166, − 106.0403666), Red Tank, Hudspeth Co., TX, USA (30.7303083, − 104.9891083), Peccary Tank, Hudspeth Co., TX, USA (30.7555556, − 105.0041667), Behind Ranch House playa, Hueco Tanks State Park and Historic Site, El Paso Co., TX, USA (31.924072, − 106.041589), Laguna Prieta rehydrated sediments, Hueco Tanks State Park and Historic Site, El Paso Co., TX, USA (31.9246388, − 106.046675), Paint Gap Cattle Tank, Big Bend National Park, Brewster Co., TX, USA (29.3878555, − 103.302675), Alazan Lake, Alazan Bayou Wildlife Management Area, Nacogdoches Co., TX, USA (31.5033389, − 094.7546583), Ojo de Santa Maria, Chihuahua, Mexico (31.1552777, − 107.3172222).

Morphology: Body is oval. Lorica is truncated at the anterior and rounded at the posterior part (Fig. 4). Cross-section of the lorica is arc of circle. Foot is slender with two joints, toes are parallel-sided with sharp tip. Trophi has four stout unci teeth, with inner denticulate comb at the tip of rami. Fulcrum is club shaped (Fig. 2). Dorsal plate length 150 to 230 μm.

Ecology and distribution: In our study, this species was found in various habitats including lakes, lagoons, artificial temporary tanks, temporary playas in Texas and Mexico. pH ranged from 8.4 to 9.3, temperature

ranged from 23.6 to 34.3°C and conductivity ranged from 137 to 988 μS/cm.

Euchlanis floridensis sp. nov.

urn:lsid:zoobank.org:act:E0CEDD30-5CAA-4A78-ACC2-AF8DB1D77CE5

Type locality: Krome pond, Miami-Dade Co., FL, USA, 25.883615, − 80.484920, 3.3 m.

Diagnosis: This species is distinguished from other cryptic species of *Euchlanis dilatata* complex in this study based on following character states in the ITS region: 29T; 54T; 55T; 92C; 180C; 186G; 189G; 232T; 233C; 259A; 260C; 264T; 277T; 278G; 288A; 307G; 312T; 314A; 344C; 369T; 657G; 693G; 70A; 702A; 708G; 756C.

Holotype: An amictic female from cryptic species 'F' on a permanent microscopic slide is deposited in the National Museum of Natural History, Smithsonian Institution (USNM 1532156). Paratypes (one permanent microscopic slide of amictic females, and approximately 30 amictic females preserved in 95% ethanol) are deposited in the National Museum of Natural History, Smithsonian Institution (USNM 1532157 and USNM 1532158, respectively) and UTEP Biodiversity Collections (UTEP:Zoo:129). ITS region and COI gene sequences are deposited in GenBank (accession numbers, ITS: KU665985 and KX714931, COI: KU665896 and KX714921).

Etymology: The species named refers to the state of Florida, USA where the type specimen was collected.

Other examined populations: Buttrill Springs, Big Bend National Park, Brewster Co., TX, USA (29.54585, − 103.2738), Cattail Falls, pool C', Big Bend National Park, Brewster Co., TX, USA (29.2731833, − 103.3361638), Cattail Falls, pool D, Big Bend National Park, Brewster Co., TX, USA (29.2731527, − 103.3358277), Cattail Falls, pool E, Big Bend National Park, Brewster Co., TX, USA (29.2731444, − 103.3361638), Cattail Falls, pool G, Big Bend National Park, Brewster Co., TX, USA (29.2731666, − 103.3361638), Cattail Falls, pool H, Big Bend National Park, Brewster Co., TX, USA (29.2731694, − 103.3362388), Duck Pond, Beaver Co., OK, USA (35.551944, − 97.574722), Lake June in the Winter, Highlands Co., FL, USA (27.30715, − 81.37542), Lake Jackson, Highlands Co., FL, USA (27.48775, − 81.47664).

Morphology: Body is oval. Lorica is truncated at the anterior and rounded at the posterior part (Fig. 4). Cross-section of the lorica is arc of circle. Foot is slender with two joints, toes are parallel-sided with sharp tip. Trophi has four stout unci teeth, with inner denticulate comb at the tip of rami. Fulcrum is club shaped (Fig. 2). Dorsal plate length 98 to 199 μm.

Ecology and distribution: In our study, this species was found in a variety of habitats that includes ponds, permanent lakes, and natural springs in Texas, Florida, and Oklahoma. pH ranged from 5.9 to 7.9, temperature ranged from 7.5 to 22.6°C and conductivity ranged from 405 to 523 μS/cm.

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