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RESEARCH ARTICLE

Intraspecific variation in sensitivity to food availability and temperature-induced phenotypic plasticity in the rotifer *Keratella cochlearis*

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ABSTRACT

Organisms with wide environmentally induced morphological plasticity and cosmopolitan distribution, e.g. the common freshwater rotifer *Keratella cochlearis*, are ideal models to study the evolution of plastic polymorphisms and the capacity of zooplankton to adapt to local selection conditions. We investigated population-level differences (population-by-environment interaction) in sensitivity to food availability and temperature-induced phenotypic plasticity between two clones of *K. cochlearis* isolated from neighboring populations in Ruidera Natural Park (Spain) with different trophic statuses: Tinaja lake (mesotrophic) and Cueva Morenilla lake (eutrophic). Using common-garden experiments, each clone proved to have a different sensitivity to food availability, with substantial phenotypic differences between them. When rotifers grew at moderate temperature (15.6°C), low food levels were more efficiently used by the Tinaja versus Cueva Morenilla clone, whereas high food levels were more efficiently used by the Cueva Morenilla versus Tinaja clone. The posterior spine was much longer and the lorica wider in the Tinaja versus Cueva Morenilla clone, with no difference in lorica length. Phylogenetic analysis based on cytochrome *c* oxidase subunit I (COI) gene sequences showed that the two populations have the same haplotype. This is the first study to show possible local adaptation by a rotifer species to habitats that consistently differ in food availability. We also detected an intriguing deviation from the expected negative relationship between posterior spine length and temperature. Our experimental results indicate that intermediate temperatures may activate the gene responsible for spine elongation in *K. cochlearis*. This suggests that rotifers in nature could use water temperature as proxy signal of a change in predation risk before defense is needed.

KEY WORDS: Population-by-environment interaction, Proxy cue, Spine elongation, Trade-off

INTRODUCTION

Phenotypic plasticity is a key element in the functioning of organisms in variable environments, and influences their evolution (e.g. Dewitt and Scheiner, 2004a). A common definition of phenotypic plasticity is the environmentally sensitive production of alternative phenotypes by given genotypes (Stearns, 1989; Dewitt and Scheiner, 2004b). The environmental factors may be either abiotic (e.g. temperature and salinity) or biotic (e.g. food concentration and predation risk). Phenotypic plasticity can be visualized using the so-called reaction norm (or performance curve), usually in a graph plotting the phenotype value of a genotype against a changing environment (Stearns, 1989). It is defined by a reaction norm that deviates from a flat line parallel to the environmental axis (e.g. Pigliucci, 2001).

A major goal of evolutionary ecology is to detect genetic variation in the phenotypic plasticity of fitness-related traits, which is essential to facilitate local adaptation to environmental changes (e.g. Dewitt and Scheiner, 2004a). Genetic variation may also reveal the phenotypic trade-offs between fitness-related traits due to resource allocation (e.g. Mazer and Damuth, 2001), a crucial concept for theoretical and empirical research on local adaptation (Kawecki and Ebert, 2004). Common-garden experiments are adequate to study the evolution of fitness-related traits (e.g. intrinsic population growth rate) and to determine the trade-offs and relative costs of local adaptation in populations (e.g. Campillo et al., 2009). In these experiments, detection of the interaction between population and environment is important because it is an evident pre-requisite for local adaptation (Kawecki and Ebert, 2004). This interaction means that the response to an environment condition differs among populations (e.g. Dewitt and Scheiner, 2004b). In such cases, reaction norms will be non-parallel and the relative fitness of populations will change with the changing environment, also indicating genetic variation in phenotypic plasticity. However, if the reaction norms are parallel, the populations react to a change in conditions, exhibiting plasticity, but this change has no effect on their differential fitness.

Monogonont rotifers are model organisms for studying the evolution of plastic polymorphisms in zooplankton (reviewed by Gilbert, 2013, 2017; Declerck and Papakostas, 2017). However, their potential for rapid ecological adaptation remains less explored in eco-evolutionary studies in comparison to other model organisms such as cladocerans (reviewed in Declerck and Papakostas, 2017). A limited number of experimental studies have yielded evidence on local adaptation of rotifers in relation to salinity (Campillo et al., 2009, 2011; Alcántara-Rodríguez et al., 2012; Scheuerl and Stelzer, 2013) and nutrient limitation (Declerck et al., 2015). All of these studies were conducted in two taxa of a single genus, the cryptic species complexes *Brachionus calyciflorus* and *Brachionus plicatilis*, which are readily cultured and manipulated. Unfortunately, no empirical data are available on the adaptation to local conditions of genera other than *Brachionus*. There has also been no published research on the intraspecific variation in sensitivity to food availability of rotifer populations from habitats that consistently differ in food concentration.

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Resource limitation is a key factor in the structuring of plankton communities (Gliwicz, 1985) and is one of the most important environmental factors affecting rotifers (González and Frost, 1992; Merriman and Kirk, 2000). Genetic variation among planktonic grazers in resource acquisition capacity constitutes a general adaptive trade-off in the ability to exploit rich versus poor food conditions (e.g. Stemberger and Gilbert, 1985; Tessier et al., 2000). This fundamental trade-off in resource exploitation in zooplankton has been positively associated with body size in herbivorous rotifers (Stemberger and Gilbert, 1985), indicating an energetic advantage for smaller versus larger rotifers when food levels are consistently low but not necessarily when food levels are high. Stemberger and Gilbert (1987) suggested that the smallest rotifers have the lowest threshold food levels (hereafter TFLs) for zero population growth because of their higher mass-specific swimming speed. However, TFLs are not species-specific constants but rather change along environmental gradients such as temperature (Stelzer, 1998), affecting species performance and possibly modifying the above trade-off (Tessier et al., 2000). In addition, species (and probably populations within species) might deviate from this positive relationship between rotifer body size and TFL through special adaptations to lower food levels (e.g. spine elongation) that reduce their sinking rate and therefore the energetic cost of maintaining their position in the water column (Stemberger and Gilbert, 1985; Gilbert, 2017, 2018). Spine elongation also reduces the vulnerability of rotifers to invertebrate predators (reviewed in Gilbert, 2013). However, it has also been proposed that the swimming speed and clearing rates of rotifers may be constrained by ciliated coronal areas (Stemberger and Gilbert, 1987; Diéguez et al., 1998; Fontaneto and Ricci, 2004).

Keratella cochlearis (Gosse 1851) is one of the most common planktonic rotifers (Segers and De Smet, 2008) and may be the most common freshwater metazoan worldwide (Lindström and Pejler, 1975). It is one of the smallest rotifer species and has the lowest TFL (Stemberger and Gilbert, 1987), while its instantaneous rate of population increase (r_m) responds rapidly to small rises in food level (Stemberger and Gilbert, 1985; Ramos-Rodríguez and Conde-Porcuna, 2003). *Keratella cochlearis* appears to ingest a wide range of particles. Feeding studies with different algal types or with natural phytoplankton mixtures have established the importance of flagellated algal cells, such as cryptomonads and chrysomonads, in its diet (Gilbert and Bogdan, 1981), although it may eat detritus, bacteria and picoplanktonic algae as an additional food resource (Pourriot, 1965; Ronneberger, 1998). Moreover, *K. cochlearis* is generally regarded as a thermal generalist (i.e. eurythermal) species (Herzig, 1983; May, 1983; Andrew and Fitzsimons, 1992; Jersabek and Bolortsetseg, 2010), and its optimal temperature has been described as 15°C according to the maximum abundance recorded in field observations (Bērzinš and Pejler, 1989) and laboratory cultures (Walz, 1983, 1987; Meyer et al., 2017). If there were a thermal specialist–generalist trade-off (Dam, 2013) in *K. cochlearis*, a generalist genotype would tolerate a wider range of temperatures but would have a lower performance at its optimal temperature in comparison to a specialist genotype. Because of the cosmopolitan distribution of this species and the wide variability in its environmental conditions (e.g. temperature, food availability and predation risk), it is has become a model organism for research into the environmentally induced morphological plasticity of lorica size and spine elongation (see review in Gilbert, 2017). *Keratella cochlearis* is efficiently preyed upon by the large predatory rotifer *Asplanchna* (e.g. Conde-Porcuna and Sarma, 1995), by cyclopoid and calanoid copepods (e.g. Ramos-Rodríguez and Conde-Porcuna, 2004), by insect larva *Chaoborus flavicans*, and by small fish (Hewitt and George, 1987; Zhang et al., 2017a,b). More recently, DNA taxonomy studies revealed that *K. cochlearis* is a complex of cryptic rotifer species, and the differentiation of taxa is only partially supported by morphological differences in their lorica (Derry et al., 2003; Cieplinski et al., 2017). For instance, *K. cochlearis* is a complex of putative evolutionarily significant units (ESUs) in which spined and unspined specimens occur in the same ESUs and in different ESUs (Cieplinski et al., 2017). Therefore, the presence and length of the posterior spine in *K. cochlearis* appears to offer poor discriminatory capacity for this complex (Cieplinski et al., 2017). Moreover, mitonuclear discordance in the delimitation of ESUs was recently reported for *K. cochlearis* species complex (Obertegger et al., 2018). Therefore, the identification of ESUs based on DNA sequences alone is problematic, and usually requires morphological, demographic and/or ecological evidence (Cieplinski et al., 2018; Obertegger et al., 2018). However, despite ample genetic variation among genotypes and populations, there has been no published research on the local adaptability of this species complex.

The plasticity of the posterior spine length (PSL) in *K. cochlearis* has been associated with three environmental factors: chemical cues (kairomones) released by both invertebrate predators and fish larvae; food concentration; and temperature (reviewed in Gilbert, 2017). The three factors are interrelated in nature, and their spine-promoting effects in rotifers can be additive for maximal spine development (Gilbert, 2017). Furthermore, various morphotypes of *K. cochlearis* have shown different tolerance to temperature and trophic state (Bērzinš and Pejler, 1989). For these reasons, it can be difficult to evaluate the relative importance of each factor at any given time in field samples. The PSL and lorica size of *K. cochlearis* can also affect fitness and competitive ability. There is a general consensus from field observations that *K. cochlearis* tends to be longer and have longer spines in lakes with lower versus higher food availability (Hillbricht-Ilkowska, 1972; Green, 2007; Gopko and Telesh, 2013). However, the specific effect of temperature could not be controlled in these studies. As noted above, longer spines in rotifers lessen the energetic cost of staying in position by reducing the sinking rate and swimming costs (Zagarese and Marinone, 1992; Stemberger, 1990). There is also agreement that *K. cochlearis* is larger (Green, 1998; Gilbert, 2017) and has longer spines at lower temperatures, in accordance with the temperature–size rule (TSR) (Atkinson, 1994). This rule has been widely documented in seasonal studies of natural populations of *K. cochlearis* (Lauterborn, 1900; Lindström and Pejler, 1975; Eloranta, 1982; Conde-Porcuna et al., 1993; Bielañska-Grajner, 1995; Green, 1998, 2005; Czarnoleski et al., 2015), leading to the general assumption of a negative linear relationship with water temperature in oxygenated water. In anoxic water, *K. cochlearis* have a shorter lorica length regardless of temperature (Czarnoleski et al., 2015). However, no investigation of the specific effect of temperature has considered a wide temperature range or controlled for other factors that influence spine length variation (e.g. the presence of kairomones from predators and food limitation in well-oxygenated water). Besides, although most ectotherms follow the general rule that hotter is smaller (Atkinson, 1994), a growth pattern that does not follow the temperature–size rule is favored in cladocerans under certain conditions, related to the action of temperature as a cue that signals a change in predation risk (e.g. Miehls et al., 2013). To the best of our knowledge, only one experimental study has shown that temperature directly induces spine elongation in *K. cochlearis* (Lindström and Pejler, 1975). However, it used two extreme

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temperatures (5 and 20°C), and no data are available on the effects on *K. cochlearis* clones of intermediate temperatures, known to be optimal for the performance of this species (Walz, 1983; Bērziņš and Pejler, 1989). It is also possible that the effects of temperature on spine elongation may differ among clones of the same rotifer species, as observed by Athibai and Sanoamuang (2008) in their study of long- and short-spined clones of *Brachionus caudatus*.

In the present study, we performed two common-garden experiments to investigate the phenotypic plasticity of two clones of *K. cochlearis* to food availability and temperature. They were isolated from neighboring populations that mainly differed in trophic status. We also carried out a phylogenetic analysis based on cytochrome *c* oxidase subunit I (COI) gene sequences to estimate the degree of genetic differentiation between the populations. The main objectives of our experiments were: (1) to test for genetic variation in fitness reaction norms to food availability between the two clones (i.e. clone–food quantity interaction); (2) to evaluate changes in population growth and TFL along a temperature gradient; and (3) to determine whether the two clonal populations differed in the temperature-induced phenotypic plasticity of adaptive morphometrical traits (lorica length, lorica width and PSL). Further objective were to explore the possible local adaptation to low food levels in these populations and to determine whether possible differences in body size and/or posterior spine elongation between the two *Keratella* clones were associated with their competitive ability to exploit resources. The study hypotheses were: (1) the adaptation of these populations to food limitation would reduce their sensitivity to a decline in food quantity; and (2) individuals with longer spines and wider lorica would be observed in *K. cochlearis* populations that inhabit food-poor environments if a long posterior spine and a wide lorica are adaptive traits for low food availability.

MATERIALS AND METHODS

Collection sites

Keratella cochlearis clonal lineages were established in our laboratory in Autumn 2010 from two warm monomictic lakes with contrasting limnological characteristics: Tinaja lake and Cueva Morenilla lake (Table 1). These lakes are located in the Ruidera National Park (central Spain) and separated by 8 km. Ruidera National Park is a karst landscape formed by 15 chain-connected lakes with SE–NW flow-through and fed by groundwater. Their mixing dynamics are very rapid because of their small size (Alvarez-Cobelas et al., 2006). When groundwater levels are high, the lakes are fed by surface drainage from upper to lower lakes. We selected these two lakes because they vary substantially in size, maximum depth, mean annual temperature, temperature range, pelagic fish biomass and trophic status (Table 1), and are both inhabited by *K. cochlearis* (Moreno, 2017). Tinaja lake has a higher elevation, larger surface area and greater maximum depth in comparison to Cueva Morenilla lake. Lake trophic status, characterized by average annual values of chlorophyll-*a*, total phosphorus and Secchi disk visibility, is considered mesotrophic for Tinaja lake and eutrophic for Cueva Morenilla lake (Table 1). Cueva Morenilla lake receives water from the upper lake through a waterflow formed by a waterfall (El Hundimiento), which is also inhabited by *K. cochlearis* (Moreno, 2017).

Water renewal is a key process in the functioning of these Mediterranean lakes (Alvarez-Cobelas et al., 2006), where the high interannual variability in water availability affects limnological variables such as oxygen concentration. Therefore, at least in years with more water availability and shorter retention times, the hypolimnetic oxygen during stratification would be similar in the two lakes (M. Alvarez-Cobelas, personal communication). However, when there is less water availability and retention times are longer, hypolimnetic oxygen could be higher in Tinaja lake than in the same layer in Cueva Morenilla lake because the latter is eutrophic. We do not know whether there is hypoxia in Cueva Morenilla lake in all summers with longer retention times. Regardless, *K. cochlearis* abundance in Cueva Morenilla lake and Tinaja lake is low in summer (<1 individuals l^{-1}) (J.M.C.-P., unpublished data); therefore, *K. cochlearis* is unlikely to experience hypoxia in these Ruidera National Park lakes.

Table 1. Environmental data on the lakes from which clones of *Keratella cochlearis* were isolated

	Tinaja lake	Cueva Morenilla lake
Geographic location	38°56′N, 2°50′W	38°59′N, 2°54′W
Surface area (ha)	11.1[4]	8.8[4]
Altitude (m above sea level)	842[3]	772[3]
Maximum depth (m)	19[2]	8[2,3]
Mean annual temperature (°C)*	14.6[1]	16.2[1]
Annual temperature range (°C)*	8.3–21.4[1]	6.5–24.8[1]
Mean annual total phosphorus (µg l^{-1})*	9.33[1], ∼8[3], 4.27[4]	13.56[1], 15[3], 14.86[4]
Mean annual chlorophyll-*a* (µg l^{-1})*	2.23[1], ∼1.5[2,3], 1.08[4]	2.79[1], ∼7.0[2,3], 4.84[4]
Mean annual secchi depth (m)	7–8[3], 6.8[4]	2–3[3], 1.9[4]
Trophic state‡	Mesotrophic	Eutrophic
Pelagic fish biomass (gpue)§	6479.2[5]	10,050.4[5]

*Data are means from profiles in the water column. ‡Trophic state according to the Organization for Economic Co-operation and Development (OECD, 1982). §Pelagic fish biomass from gill net catches (gpue, grams per unit effort). [1]J.M.C.-P., E.M., E.R.-R., L. Jiménez and C. Pérez-Martínez (unpublished data), from May 2008 to November 2010 (*n*=13–15; Comisión Interministerial de Ciencia y Tecnología project: CGL2007-65784/BOS); [2]Bort et al. (2005), from May 2000 until December 2001 (*n*=6); [3]Alvarez-Cobelas et al. (2006), between 2000 and 2001 (*n*=8); [4]J.M.C.-P., E.R.-R., J. Medina-Sánchez and C. Pérez-Martínez (unpublished data) from May 2000 to October 2000 (*n*=6, EU project: Biodiversity and human impact in shallow lakes). [5]J.M.C.-P., E.R.-R., J. Medina-Sánchez and C. Pérez-Martínez (unpublished data), on July 2000 (EU project: BIOMAN, EVK2-CT-1999-00046).

A taxonomic study of phytoplankton in the lakes of Ruidera Natural Park in 2000–2001 revealed that the temporal dynamics of phytoplankton abundance and composition differed between Tinaja and Cueva Morenilla lakes (Alvarez-Cobelas et al., 2007). In spring and autumn, when *K. cochlearis* is more abundant in these lakes (Moreno, 2017), a higher biovolume of edible phytoplankton (cryptophyceae, chrysophyceae and chlorophyceae groups) was estimated in Cueva Morenilla lake than in Tinaja lake (Alvarez-Cobelas et al., 2007). Moreover, a higher mean annual bacterial abundance was found in Cueva Morenilla than in Tinaja lake, where autotrophic picoplankton was undetectable (Alvarez-Cobelas et al., 2007).

The composition and biomass of zooplankton species change seasonally and differ between the lakes, with rotifers, especially *Asplanchna*, *Polyarthra*, *Synchaeta pectinata* and *K. cochlearis*, being more abundant in Cueva Morenilla lake (Moreno, 2017). In both lakes, the most abundant taxa of cladocerans and copepods are the small-bodied bosminid *Bosmina longirostris* and the small predator cyclopoid *Tropocyclops prasinus*, respectively. The maximum abundance of adults and copepodites of *T. prasinus* has been recorded as 8.5 individuals l^{-1} in Tinaja and 4.5 individuals l^{-1} in Cueva Morenilla (Moreno, 2017). Cladocerans are potential competitors with rotifers by mechanical interference and exploitative competition (reviewed in Gilbert,

1988). However, *K. cochlearis* has a low susceptibility to interference competition by *Bosmina longirostris* (Gilbert and MacIsaac, 1989) because of the latter's small body size (<1.2 mm). *Tropocyclops prasinus* is a potential predator on rotifers that may play a crucial role in inducing posterior spine development in *K. cochlearis* (Stemberger and Gilbert, 1984). Nevertheless, the spine enlargement in *K. cochlearis* can be directly related to the abundance of predator copepods (e.g. Baião et al., 1999). Because the mean annual abundance of *T. prasinus* is very low in Ruidera lakes (<2 individuals l^{-1}; Moreno, 2017), the cyclopoid predation pressure on spine enlargement in *K. cochlearis* could be considered negligible.

Culture conditions and stock populations

Two clonal stock cultures of *K. cochlearis* were established from a single female isolated from plankton samples from each lake in oxygen-saturated mineral water. Stock cultures were maintained in an isolated room (6 m^2) at a controlled temperature of 16°C with ∼100 µmol quanta m^{-2} s^{-1} photosynthetically active radiation (PAR) on a 14 h:10 h light:dark cycle (Fig. 1). They were fed *Cryptomonas pyrenoidifera* Geitler cells (CCAP 979/61) (∼1 mg C l^{-1}) once a week. The genus *Cryptomonas*, rich in highly unsaturated fatty acids (Ahlgren et al., 1990), is food of excellent quality for *K. cochlearis* (e.g. Ramos-Rodríguez and Conde-Porcuna, 2003), and a concentration of 1 mg C l^{-1} is sufficient to support the maximal growth rate of rotifers (Stemberger and Gilbert, 1985). *Cryptomonas pyrenoidifera* (750 µm^3; 15.97 µm×9.38 µm) was routinely cultured at our laboratory in a chemostat (dilution rate: ∼0.1 day^{-1}) in an isolated room at 16°C with sterile-filtered air and ∼100 µmol quanta m^{-2} s^{-1} PAR on a 14 h:10 h light:dark cycle in Bold Modified Basal Freshwater Nutrient Solution (BBM; B5282 Sigma-Aldrich). Although the *C. pyrenoidifera* culture was not axenic, it was unialgal and free of protozoan and fungal contaminants, and sterile techniques were always used. Under these culture conditions, the carbon content of one *C. pyrenoidifera* cell was 104±15 pg (mean±s.d., n=3; mean molar C:N:P=130:16:1). The elemental composition of the microalgae was determined by filtering a cell suspension on precombusted (460°C, 4 h) glass-fiber filters (Whatman GF/F), measuring particulate C and N with a CNH analyzer (Perkin Elmer) and determining particulate P as soluble reactive P after potassium persulfate digestion (Murphy and Riley, 1962). Three measurements were performed in each analysis. Cell density was estimated with an improved Neubauer hemacytometer.

Common-garden experiments

Experiment 1: fitness reaction norms

This experiment was designed to examine variations in fitness reaction norms of r_m to food quantity between the two *K. cochlearis* clones (clone–food interaction) and changes in their r_m and TFL along a temperature gradient (see Fig. 1). We hypothesized that, if the populations were locally adapted to food limitation, a lesser sensitivity to a decline in food quantity and lower TFL would be observed for the clone isolated from Tinaja lake, where the food availability (chlorophyll-*a*) is lower than in Cueva Morenilla lake (Table 1), and a greater sensitivity to poor food conditions and higher TFL would be observed in the clone from Cueva Morenilla lake. We simultaneously measured the r_m of *K. cochlearis* clones from each population at combinations of three different temperatures (9.7, 15.6 and 18.5°C) with four food levels [250, 1000, 3000 and 6000 cells ml^{-1} (0.026, 0.104, 0.312 and 0.624 mg C l^{-1}) of *C. pyrenoidifera*], using daily renewed batch cultures. For another *K. cochlearis* clone fed on *Cryptomonas* sp., the mean (±s.d.) TFL was reported to be 0.018±0.004 mg C l^{-1} at 20°C (Ramos-Rodríguez, 2003). The r_m of *K. cochlearis* was considered as a measure of the short-term performance of a clone in the exploitation of a given food environment (Tessier et al., 2000; Declerck and Papakostas, 2017). This design required 72 experimental cultures (2 populations×3 temperatures×4 food concentrations×3 replicates).

Memmert incubators were used for experiments at 9.7°C (ICP 700, designed specifically for low temperatures) and 18.5°C (ICP 600) and the thermally isolated room for an experiment at 15.6°C. The same light environment conditions were maintained in the incubators and room (90–120 µmol quanta m^{-2} s^{-1}). The temperatures initially contemplated in our experimental design were 12, 16 and 20°C. We recorded the minimum and maximum temperatures during experiments using a minimum/maximum thermometer, establishing that the mean (±s.d.) experimental temperatures were 9.7±0.8, 15.6±0.9 and 18.5±0.9°C. These experimental temperatures can be observed in the two lakes during the time that *K. cochlearis* is present (Moreno, 2017).

Individuals were acclimated to lower food conditions than those in stock cultures by placing 400 animals isolated from stock cultures into 500 ml Erlenmeyer flasks. In these pre-experimental cultures, temperature and photoperiod conditions were similar to those of stock cultures, but the animals were fed on ca. 0.2 mg C l^{-1} *C. pyrenoidifera* every 2 days. Rotifers were acclimated to this intermediate food level over a 6 day period.

Experimental cultures were started in March 2011 by randomly transferring 5 individuals without eggs from the corresponding pre-experimental cultures to each of three replicate containers per treatment; the containers were 5 ml wells in disposable, 12-well polystyrene tissue-culture plates. Rotifers were counted and transferred daily into new containers for 19–32 days using a stereomicroscope (25×) and sterile mouth pipette. In order to prevent density dependence effects during the 11–12 day period of acclimation to different temperatures, a maximum of 10 rotifers were cultured per well (i.e. 2 individuals ml^{-1}, as in Stelzer, 1998). This is an important assumption for validating r_m estimations (Caswell, 1989). When this number was exceeded, 10 rotifers were randomly selected from the acclimated populations and transferred into new test wells. Male neonates (maximum of one per day) were observed in some replicates after the acclimation period but were eliminated, and 10 individuals were again randomly isolated from the corresponding replicate and counted for 9 more days. No mictic females carrying resting eggs were observed. Food for rotifers was prepared from algae concentrated by centrifugation (3000 rpm, 5 min) and diluted with mineral water to yield the desired food concentration. Bacterial abundance was estimated in the food suspensions by fluorescence microscopy. Given that the maximum bacterial biomass was always <1% of the TFL of these *K. cochlearis* clones, bacterial contamination did not significantly contribute to the nutrition of these rotifers.

The r_m values were estimated by regressing $\ln(N_i/N_0)$ against time, where N_0 is initial rotifer density and N_i is population density on day i. The slope of the resulting regression line is the instantaneous population growth rate, r_m, or the intrinsic rate of natural increase per day. N_0 was the population in each well after 11–12 days of growth, when a stable age distribution was achieved and r_m values became relatively constant, which is essential for the precise estimation of r_m (Caswell, 1989). Other studies observed the acclimation of rotifers to temperature after 2 weeks (Stelzer, 1998) and to food concentration after only 3–4 days (Stemberger and Gilbert, 1985; Stelzer, 1998).

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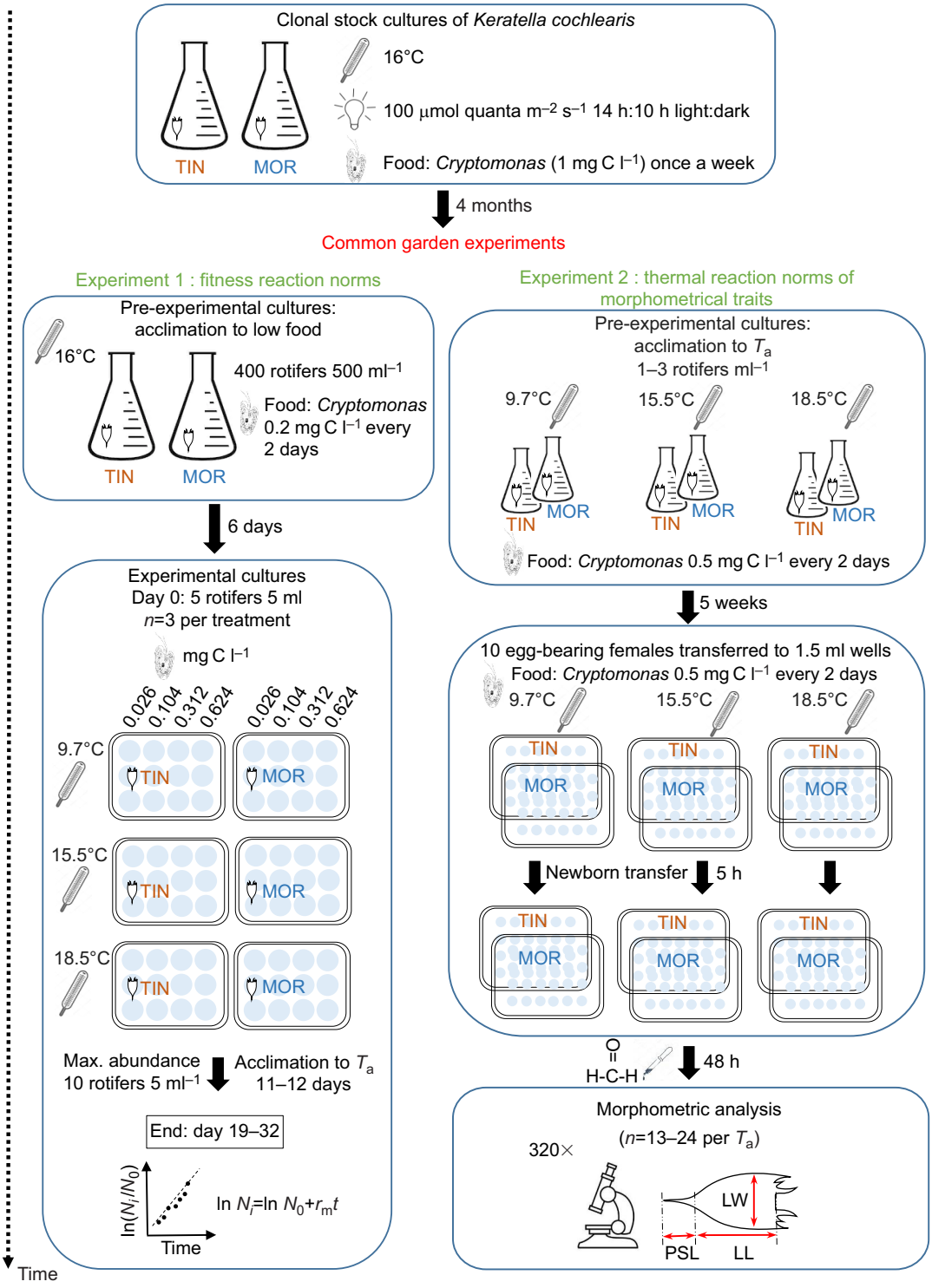

Fig. 1. Schematic diagram of the experimental set-up. For details, see Materials and Methods. TIN, Tinaja lake; MOR, Cueva Morenilla lake. N_0, initial rotifer density; N_i, population density on day i; PSL, posterior spine length; LL, lorica length; LW, lorica width.

We did not measure the morphometrical traits of the rotifers in this experiment because their measurement is preferentially performed at a given stage (e.g. neonates or adults) (Gilbert, 2013).

Experiment 2: thermal reaction norms of morphometrical traits

Preliminary observations of rotifer stock cultures indicated that the PSL may be one of the main differences between the two clonal populations of *K. cochlearis*. Hence, the second experiment was designed to determine whether the two populations differed in the temperature-induced phenotypic plasticity of adaptive morphometrical traits and to examine the direct effects of temperature on lorica size (length and width) and PSL in this rotifer species (see Fig. 1). A further aim was to establish whether possible differences in body size and PSL between these populations were associated with their competitive

ability to exploit resources as observed in experiment 1. In experiment 2, three pre-experimental cultures per clone were established in April 2011 and grown independently in 100 ml of mineral water at 9.7, 15.6 and 18.5°C (one incubator per temperature), and fed 0.5 mg C l^{-1} of *C. pyrenoidifera*, which exceeds the TFLs of our *K. cochlearis* clones. This design required six pre-experimental cultures (2 populations×3 temperatures). The cultures were inspected once a week to maintain the density of animals at 1–3 individuals ml^{-1} and their food concentration at ∼0.5 mg C l^{-1}. When necessary, cultures were diluted with fresh culture medium or fed with a concentrated *C. pyrenoidifera* suspension, as appropriate. After a 5 week acclimation period to obtain sufficient egg-bearing females, 62–240 of these were randomly transferred to 1.5 ml wells (10 females per well) and kept at the corresponding experimental temperature under standard food conditions. After 5 h, 13–24 newborns were individually transferred to new wells containing fresh medium under the same culture conditions. After 48 h, the animals, which all survived, were fixed with 4% formaldehyde. This experimental design ensured that all females had the same age class (48–53 h), reducing the variance in measurements (Gilbert, 2013). Three morphometrical traits were considered at all three temperatures: lorica length without considering anterior and posterior spine (hereafter LL), lorica width at its widest part (hereafter LW) and PSL. Morphometric analysis was done using an inverted microscope (Nikon Eclipse TE2000-S) with 32× objective lens and 10× eyepiece (maximum magnification of 320×). The length of the three pairs of anterior spines was not measured because temperature had no significant effect on these spines in *Keratella tropica* (Gilbert, 2011). The ratio of PSL to total length (hereafter relative spine length index, RSL) was calculated as a measure of posterior spine elongation.

Phylogenetic analysis

Given that *K. cochlearis* belongs to a cryptic species complex (Derry et al., 2003), specimens from each experimental clone were genetically characterized through the sequencing and analysis of a DNA fragment of the COI gene. We constructed a dataset that included all available *K. cochlearis* COI sequences downloaded from GenBank and four new sequences obtained from this study (two from Cueva Morenilla lake and two from Tinaja lake). Sequences were aligned using the ClustalW algorithm in MEGA7 (Kumar et al., 2016) and trimmed to 557 base pairs, the length of the shortest sequence in our alignment. The alignment was individually checked and verified for protein coding frame shifts to avoid pseudogenes using MEGA 7 (Kumar et al., 2016). All sequences obtained in this study were submitted to GenBank with accession numbers MT103157–MT103160. Identical sequences were collapsed into haplotypes using the online fasta sequence toolbox FaBox v.1.4 (Villesen, 2007) before the phylogenetic analyses. We reconstructed the phylogenetic tree using a maximum likelihood (ML) and Bayesian inference (BI) approach. The model of evolution for the phylogenetic reconstruction was HKY+I, selected with the 'Find Best DNA/Protein Models' option in MEGA7 (Kumar et al., 2016). The selected model was implemented in MEGA7 (Kumar et al., 2016) for the ML reconstruction using the approximate likelihood ratio test to evaluate node support. For BI, we used BEAST v1.8.0 (Drummond et al., 2012) with the following settings: uncorrelated lognormal relaxed clock, HKY+I substitution model, and the coalescent model with default parameters. The probability distribution was estimated by Markov chain Monte Carlo (MCMC) sampling, which was run for 100 million generations, sampling every 10,000 generations. Tracer v1.5 (http://beast.bio.ed.ac.uk/tracer) was used to investigate the convergence and the accuracy of the MCMC model and to determine the burn-in. We used TreeAnnotator v1.7.5 to summarize trees and discard the first 2000 trees as burn-in. As outgroup sequence, we used *Brachionus* sp. Tiscar (GenBank accession number KY749386.1), a monogonont rotifer belonging to the same family (i.e. Brachionidae) as *Keratella*. We inferred GMYC groups within *K. cochlearis* using the generalized mixed Yule coalescent (GMYC) approach (Pons et al., 2006; Fontaneto et al., 2007; Fujisawa and Barraclough, 2013). The previously constructed ultrametric maximum clade credibility consensus tree was performed in R v2.14.1 (http://www.R-project.org/) with the splits v1.0-14/r31 package (https://r-forge.r-project.org/projects/splits/) for the GMYC analysis.

We adopted a second approach for delimiting species, using Automatic Barcode Gap Discovery (ABGD, http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html), which identifies a threshold in genetic distances for species delimitation (Puillandre et al., 2012). We applied ABGD analyses for our dataset with the outgroup excluded and the following default options: Pmin: 0.001, Pmax: 0.1, steps: 10, Nb bins: 20; except for the relative gap width (X), which was set at 1. We only considered results with prior intraspecific divergence >1.5%, previously described in rotifers for COI (Fontaneto, 2014).

Statistical analyses

Main statistical analyses

Statistical analyses were performed with STATISTICA (v.7.1, Statsoft) and R 3.4.3 (http://www.R-project.org/) programs. Because a single chamber was used for each temperature, the three temperature treatments were not truly replicated (Hurlbert, 1984); therefore, all analyses of rotifer reaction norms were performed separately for each temperature.

To examine whether the two *K. cochlearis* populations differed in their response to the array of food levels (lake–food interaction), we first analyzed the r_m data using a general linear model (GLM) for each experimental temperature, in which lake was treated as a categorical variable and food quantity (log-transformed) as a continuous variable. The Kolmogorov–Smirnov normality test of standardized residuals, variance-inflation factor multicollinearity, Breusch–Pagan test for heteroscedasticity, Durbin–Watson test for autocorrelation, RESET test for non-linearity and Bonferroni outlier test were used to check the fit of data to linear model assumptions. We removed the interaction term (lake × food level) from the model if it did not reach significance in order to check changes in the significance of main effects, given that some authors drop non-significant interactions for this purpose (Pinheiro and Bates, 2000; Bolker et al., 2009).

The Friedman test and paired *t*-test were applied to compare r_m values between the three matched temperature groups of each *K. cochlearis* population. The normality of variable distribution was checked with the Kolmogorov–Smirnov test. The false discovery rate (FDR; Benjamini and Hochberg, 1995) was used to determine the threshold of *P*-values in all of these tests.

Differences in the sensitivity to food concentrations between the *K. cochlearis* populations were examined at each temperature. For this purpose, the fitness reaction norms of r_m were depicted as linear growth responses of the two clones to the food quantity gradient. The sensitivity of r_m to a decline in food availability was evaluated as the regression slope of r_m on the resource values estimated as the mean r_m of both clones at each food concentration (i.e. the environmental value; Falconer and Mackay, 1996), as in Tessier et al. (2000). Differences between slopes (lake × mean resource

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value interaction) were evaluated by means of a GLM analysis for each experimental temperature.

In addition, differences between the clones in their growth efficiency at low food concentrations were evaluated according to their TFL at each temperature. The Monod model with a threshold for zero-growth was used to describe the relationship between population growth rate (r_m) and food concentration (C, mg C l^{-1}), defined by Eqn 1:

$$r_m = r_{max}\frac{C - \mathrm{TFL}}{C - \mathrm{TFL} + K_s}, \quad (1)$$

where r_{max} is the maximal population growth rate, TFL is the threshold food level for zero population growth and K_s is the Monod constant. Fitting was computed by the non-linear least-squares method. Although all fitness reaction norms showed a good fit of the modified Monod equation with the food level (P-levels of the regression <0.05), estimates of the TFL for Tinaja lake at 9.7 and 15.6°C were not significant (P>0.05) and a negative value was recorded for Cueva Morenilla lake at 9.7°C. Therefore, the TFLs of these cases were graphically estimated as the intersection of the standard error bar of r_m with the r_m=0 line (Cueva Morenilla lake, 9.7°C) or by drawing a line between the negative and positive r_m values closest to zero (Tinaja lake, 9.7 and 18.5°C), as in Stemberger and Gilbert (1985). When comparisons were possible, regression parameters of each clonal population were considered statistically significant when the 95% confidence intervals did not overlap (Sachs, 1992).

Differences in morphometrical traits of *K. cochlearis* between the two clonal populations were examined by applying the Mann–Whitney *U*-test separately for each temperature, using a non-parametric test because a normal distribution of the data could not be achieved by transformation. A two-way ANOVA without replication was used to test the main effects of temperature and clone origin on morphometrical trait measurements and to avoid pseudo-replication, considering the mean value of each morphometrical trait by clone and incubation temperature. The Kolmogorov–Smirnov normality test of standardized residuals and the Breusch–Pagan test for equality of error variances were used to check the fit of data to linear model assumptions.

Additional statistical analyses

In addition to the main analyses, we also performed less conservative crossed statistical analyses to study temperature effects by using wells inside chambers as experimental units, although there would be pseudoreplication because the wells were distributed among only three temperature chambers (Hurlbert, 1984). Nevertheless, Oksanen (2001, 2004) asserted that the results of such experiments may be interpreted by combining the use of statistics with common sense when replication is difficult and when there is virtually no likelihood that local factors other than the treatment influence the experimental results. However, Cottenie and De Meester (2003) stated that this strategy was only acceptable when the authors acknowledged the limited scope of their results. We conducted crossed analyses because we considered there to be no uncontrolled factors affecting the temperature chambers. Nevertheless, the results should be interpreted with caution because there was no proper replication, and these supplementary findings are limited to the specific chambers tested (Cottenie and De Meester, 2003).

In brief, linear models were used to test whether the mean value of r_m and sensitivity to food concentration were associated with clone, food concentration (or resource values), incubation temperature and their interactions. In these models, temperature was considered as a categorical variable, while food quantity was also considered as a categorical variable but only when comparing r_m values, because differences in the sensitivity to food concentrations must be evaluated as the regression slopes of r_m on the resource values (see above). The normality of residuals was checked by the Shapiro test, and outliers were evaluated with the Bonferroni outlier test. Homoscedasticity was confirmed by the results of the Breusch–Pagan test for heteroscedasticity.

An ANOVA with replicates was also used to test whether each morphometrical trait of *K. cochlearis* was associated with clone, temperature and/or their interaction. However, no transformation of LL, LW or PSL variables allowed the models to meet the ANOVA assumptions. In consequence, a two-way analysis of variance design for ranked data (the Scheirer–Ray–Hare extension of the Kruskal–Wallis test) was performed (Sokal and Rohlf, 1995) to test the effects of lake, temperature and their interaction on these morphometrical traits.

Regardless of the aforementioned pseudoreplication problem related to these additional analyses, the results of our main statistical analyses avoid pseudoreplication.

RESULTS

Comparison of population performance across a food gradient

Linear models revealed a substantial variation in population growth rate of *K. cochlearis* caused by the food concentration gradient at all three temperatures (Table 2). As expected, r_m increased hyperbolically with food quantity (Fig. 2). However, the shape of the fitness curve varied between clones, especially at 9.7°C with the highest food concentration and 15.6°C with the lowest food concentration. This was also reflected by the linear models, in which the main effect of lake on r_m was significant at 15.6°C

Table 2. Results of linear models to test the effects of lake and food quantity (log-transformed data) on the population growth rate of *K. cochlearis* at each temperature

	9.7°C		15.6°C		18.5°C	
	Estimate±s.e.m.	*t*	Estimate±s.e.m.	*t*	Estimate±s.e.m.	*t*
(Intercept)	−0.191±0.054	−3.542**	−0.674±0.073	−9.250***	−0.564±0.109	−5.181***
Lake [TIN]	0.072±0.076	0.939^{ns}	0.445±0.103	4.317***	−0.073±0.154	−0.477^{ns}
Food quantity	0.034±0.007	4.657***	0.101±0.010	10.199***	0.090±0.015	6.108***
Lake×food quantity	−0.015±0.010	−1.434^{ns}	−0.052±0.014	−3.730**	0.006±0.021	0.308^{ns}
Observations	24		24		24	
Residual standard error	0.031 (d.f.=21)		0.041 (d.f.=20)		0.060 (d.f.=21)	
R^2/adjusted R^2	0.614/0.577		0.878/0.859		0.8016/0.7827	
F-statistic	16.71*** (d.f.=2;21)		47.86*** (d.f.=3;20)		42.41*** (d.f.=2;21)	

***P<0.001; **P<0.01; ^{ns}P>0.05. TIN, Tinaja.

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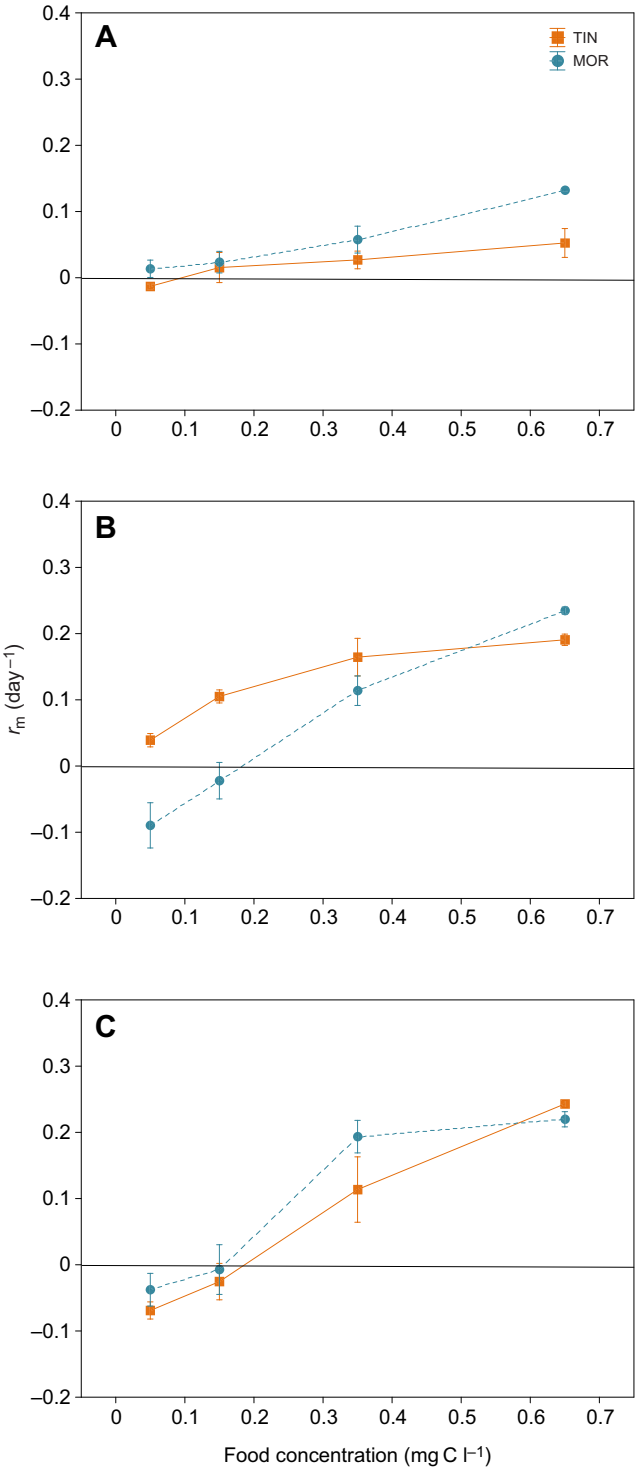

Fig. 2. Population-level reaction norms of average growth rate in response to food availability for two *Keratella cochlearis* clones at different temperatures. Data for Tinaja lake (TIN) and Cueva Morenilla lake (MOR) clones were obtained at (A) 9.7°C, (B) 15.6°C and (C) 18.5°C (experiment 1). Each data point is the mean±1 s.e.m. population growth rate (r_m) of three replicates.

(Table 2), and at 9.7°C when the non-significant interaction was removed from the model (t=−2.853, P<0.05). There was also a significant clone×food interaction at 15.6°C. The main effect of lake was not significant at 18.5°C (Table 2), although the assumption of model linearity was violated ($RESET_{2,19}$=6.49, P<0.01). Nevertheless, paired comparisons of r_m values at this temperature showed no significant difference between the lakes (t=−1.23$_{11}$, P=0.26); moreover, no differences between *K. cochlearis* clones were graphically observed (Fig. 2C).

Differences between the clones in their sensitivity to the food concentration gradient were related to the experimental temperature (Fig. 3). At 9.7 and 15.6°C, linear models indicated that the reaction norms of r_m substantially differed between clones according to the regression slopes (lake × mean resource value interaction term; Table 3). However, at 18.5°C, the two clones displayed highly similar rank preferences at the five food levels, and reaction norms of r_m of the *K. cochlearis* populations were parallel (Fig. 3C). Differences were also found in sensitivity to the food gradient at 9.7 and 15.6°C. However, although a variation in sensitivity was observed along the food gradient at 9.7°C, the rank fitness of clones did not reverse, i.e. no complete crossing of the reaction norms was observed (Fig. 3A). Thus, between-clone differences were only observed in the richest food conditions at this temperature (higher r_m for Cueva Morenilla *K. cochlearis* versus Tinaja *K. cochlearis).* At 15.6°C, however, there was a complete crossing of the reaction norms (Fig. 3B) along the food gradient, with the average relative r_m value being higher for the Tinaja versus Cueva Morenilla population in the poorest food conditions but higher for the Cueva Morenilla versus Tinaja population in the richest food conditions.

TFL estimates confirmed that between-population differences in the capacity to exploit poor food levels varied according to the temperature (Table 4). Graphically, only small between-population differences in TFL were observed at 9.7°C, with the values for both being lower than 0.1 mg C l^{-1} (Fig. 2A). At 18.5°C, although the relationships in both clones were well described by the modified Monod model, the confidence intervals overlapped. However, at 15.6°C, the TFL was markedly higher (by a factor of 4) for the Cueva Morenilla versus Tinaja clone. In addition, although the confidence interval for the TFL of Cueva Morenilla *K. cochlearis* overlapped at 15.6 and 18.5°C, the TFL of Tinaja *K. cochlearis* increased from <0.03 mg C l^{-1} at 15.6°C to 0.13 mg C l^{-1} at 18.5°C (4-fold higher).

Comparison of population performance across a temperature gradient

The effect of temperature on the r_m value differed between the populations. The Friedman test showed that it was not significant for the Tinaja population (P>0.05); however, paired comparisons with FDR-adjusted t-tests showed a significant increase in r_m values of the Tinaja clone with rising temperature, from almost zero-growth (r_m=0.020 day^{-1}) at 9.7°C to 0.125 day^{-1} at 15.6°C (t=−5.01$_3$, P=0.015). Although no significant difference in r_m was observed between 15.6 and 18.5°C, some negative r_m values were recorded at 18.5°C, whereas all r_m values were positive at 15.6°C. These results suggested a notable optimum for the Tinaja population of *K. cochlearis* at intermediate temperatures (around 15°C). However, although the r_m value of the Cueva Morenilla clone increased slightly with rising temperature, the Friedman test and paired t-tests showed no significant differences among the three temperatures (P>0.05). Thus, the growth rate of the Cueva Morenilla clone was low (0.06 day^{-1}) at 9.7 and 15.6°C and was slightly higher at 18.5°C (r_m=0.09 day^{-1}). These results and those obtained across the food gradient suggest interaction effects of lake, food concentration and temperature. When we included temperature in less conservative analyses to check for interactions among all three independent variables (see Materials and Methods), we found

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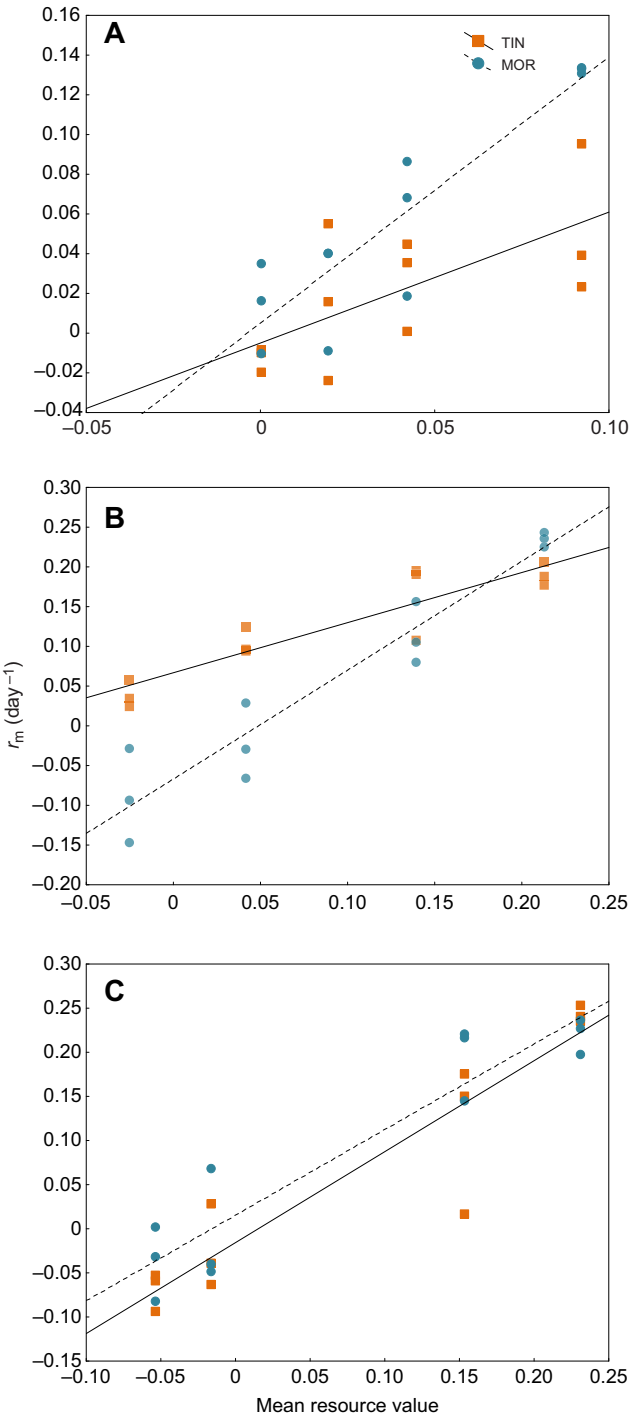

Fig. 3. Sensitivity of two *K. cochlearis* clones to food concentration at different temperatures. Population growth rates of Tinaja lake (TIN) and Cueva Morenilla lake (MOR) clones raised at three temperatures (A, 9.7°C; B, 15.6°C; C, 18.5°C) in four different food concentrations (experiment 1). Food concentrations are quantified by the mean growth rate (r_m) of the two clones (i.e. mean resource value). Lines indicate individual clone regression fits.

a significant triple interaction among factors ($F_{6,48}$=3.18, P<0.05) as well as significant interactions between lake and temperature ($F_{2,48}$=8.48, P<0.001) and between food concentration and temperature ($F_{6,48}$=6.40, P<0.001).

We had observed that differences between the clones in their sensitivity to the food concentration gradient were related to the experimental temperature. When we included temperature in less conservative analyses of sensitivity to check for interactions among all three independent variables (see Materials and Methods), we found a significant triple interaction among factors ($F_{2,59}$=11.41, P<0.001) as well as significant interactions between lake and temperature ($F_{2,59}$=22.31, P<0.001) and between mean resource value and temperature ($F_{2,59}$=4.92, P<0.05).

Comparison of morphometrical traits

Clone origin effect

Comparisons of morphological features between the populations of *K. cochlearis* from Tinaja lake and Cueva Morenilla lake revealed the same LL for both clones at each temperature [mean±s.e.m. Tinaja: 111.9±0.7 µm (n=57) versus Cueva Morenilla: 111.0 ±0.7 µm (n=60); Fig. 4A]. However, Mann–Whitney U-tests performed separately for each temperature showed that the Tinaja population had significantly wider lorica at 15.6 and 18.5°C (Fig. 4B) and a much longer spine (Fig. 4C) and higher RSL (Fig. 4D) at all temperatures in comparison to the Cueva Morenilla population. These findings verify that these clonal populations are two distinct phenotypes of *K. cochlearis*.

Temperature effect

Our laboratory study evidenced the temperature-induced phenotypic plasticity of lorica size (LL and LW) and spine elongation (PSL and RSL) in *K. cochlearis*. The two-way ANOVA without replication showed a significant main effect of temperature on LL, PSL and RSL ($F_{2,2}$=56.9, P=0.017; $F_{2,2}$=74.77, P=0.013; and $F_{2,2}$=78.02, P=0.013, respectively) but not on LW ($F_{2,2}$=2.30, P>0.05). The changes in temperature had little effect on LW in the Cueva Morenilla population but produced a much greater phenotypic variance of LW in the Tinaja population (Fig. 4B). These rotifers had the shortest lorica at the highest temperature studied (Fig. 4A). However, thermal reaction norms of body size (LL and LW) and posterior spine elongation (PSL and RSL) were concave in the Tinaja population, with a maximum LL, LW, PSL and RSL at 15.6°C (Fig. 4). Although no differences in LL and LW were observed in the Cueva Morenilla clone between 9.7 and 15.6° C, they also had a proportionally longer spine when cultured at the intermediate temperature (Fig. 4D).

Interestingly, PSL was the morphological trait that evidenced the greatest variability in *K. cochlearis*, with a coefficient of variation (CV) for all specimens that ranged from 16.5% in Cueva Morenilla lake to 17.7% in Tinaja lake. However, the CV of the LL and LW was very low (all values <5%) indicating the rather conservative character of these morphological features in *K. cochlearis.* The highest PSL value was 68.6 µm for the Tinaja lake population and 56.35 µm for the Cueva Morenilla lake population, both being observed at a temperature of 15.6°C. When we performed a two-way analysis with replicates that included the interaction between lake and temperature (see Materials and Methods), the interaction was not significant for LL ($H_{2,111}$=0.61, P=0.74), PSL ($H_{2,111}$=0.54, P=0.76) or RSL ($H_{2,111}$=0.84, P=0.66), whereas temperature was significant in the three analyses (LL: $H_{2,111}$=34.83, P<0.001; PSL: $H_{2,111}$=56.67, P<0.001; RSL: $H_{2,111}$=54.37, P<0.001). However, the interaction was significant when analyzing LW ($H_{2,111}$=6.86, P=0.03), supporting our previous observation relating to Fig. 4B.

Phylogenetic analysis

We obtained 243 sequences of the COI gene of *K. cochlearis* from GenBank in addition to our sequences, and they comprised 89 haplotypes. The evolutionary history was inferred by using the ML method based on the Tamura–Nei model (Tamura et al., 2011). The

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Table 3. Results of linear models to test the sensitivity of *K. cochlearis* populations to resource richness

	9.7°C		15.6°C		18.5°C	
	Estimate±s.e.m.	*t*	Estimate±s.e.m.	*t*	Estimate±s.e.m.	*t*
(Intercept)	0.005±0.011	0.447^{ns}	−0.067±0.014	−4.677***	0.016±0.016	0.958^{ns}
Lake [TIN]	−0.010±0.016	−0.632^{ns}	0.134±0.020	6.614***	−0.031±0.023	−1.355^{ns}
Mean resource value	1.341±0.215	6.249***	1.369±0.110	12.408***	0.969±0.116	8.359***
Lake×mean resource value	−0.682±0.303	−2.247*	−0.739±0.156	−4.734***	0.062±0.164	0.377^{ns}
Observations	24		24		24	
Residual standard error	0.026 (d.f.=20)		0.035 (d.f.=20)		0.046 (d.f.=21)	
R^2/adjusted R^2	0.752/0.714		0.912/0.900		0.882/0.871	
F-statistic	20.18*** (d.f.=3;20)		69.32*** (d.f.=3;20)		78.57*** (d.f.=2;21)	

***P<0.001; *P<0.05; ^{ns}P>0.05. TIN, Tinaja.

tree with the highest log likelihood (−2101.35) is shown in Fig. S1. The evolutionary history inferred by ML showed *Keratella cochlearis* sequences from Cueva Morenilla and Tinaja in the ESU 5 group described by Cieplinski et al. (2017) and formed an independent monophyletic group with strong support (Fig. S1). The ABGD analysis showed a clear barcode gap between 0.02 and 0.59 and grouped the sequences into six groups (Fig. S2). GMYC analysis indicated three entities (clusters plus singletons and excluding outgroup with a confidence interval 1–89: likelihood of the null model=697.35; likelihood of the GMYC approach=699.13; likelihood ratio: 3.57, LR test: 0.167). However, the groupings delimited by ABGD were incongruent with the most conservative result of the GMYC analysis. In our study, *K. cochlearis* sequences from Cueva Morenilla and Tinaja lakes were collapsed, and they belonged to the same haplotype as *K. cochlearis* sequences from Tinaja lake previously published by Moreno et al. (2017). GMYC and ABGD analyses grouped the *K. cochlearis* haplotype from Cueva Morenilla and Tinaja lakes in putative group 5 of the ESUs of the *K. cochlearis* complex described by Cieplinski et al. (2017).

DISCUSSION

Intraspecific variation in population performance

Differences in sensitivity to food availability

To our knowledge, this is the first report on intraspecific variation in the performance of rotifers in relation to food conditions, using the rotifer *K. cochlearis* isolated from habitats that consistently differ in food availability. The two clones (populations) of *K. cochlearis* substantially differed in their relative fitness at low and medium food concentrations and exhibited differences in their phenotypes.

Table 4. Summary of estimated threshold food levels (TFLs) in experiments of population-level reaction norms for growth rate in response to food availability for the two *K. cochlearis* populations

Lake	T_a (°C)	TFL (mg C l^{-1})	R^2
TIN	9.7	0.06‡	0.46
	15.6	<0.03‡	0.86
	18.5	0.13±0.03 (0.07–0.19)*; P<0.01	0.90
MOR	9.7	0.03‡	0.83
	15.6	0.13±0.02 (0.08–0.18)*; P<0.001	0.93
	18.5	0.07±0.02 (0.02–0.12)*; P<0.01	0.85

Determination coefficients (R^2) of the regressions to the modified Monod equation and *P*-level of TFL estimated by fitting to the modified Monod equation (see Materials and Methods) are shown. *Estimated parameter ±s.e.m. and 95% confidence interval (in parentheses) by fitting to the modified Monod equation; ‡TFL graphically estimated as in Stemberger and Gilbert (1985) (see Materials and Methods). All fitness reaction norms showed a good fit to the modified Monod equation with the food level (P<0.05). TIN, Tinaja; MOR, Cueva Morenilla.

Although differences between the two *K. cochlearis* clones were influenced by the experimental temperature, the significant effect on growth rate of the clone×food interaction at 15.6°C revealed a difference between these populations in their response to food availability. Substantial between-clone differences in sensitivity to the food concentration were also observed at 9.7 and 15.6°C, suggesting local adaptation in accordance with Kawecki and Ebert (2004). Under the poorest food conditions, no between-clone difference in relative fitness was observed at the lowest and highest temperatures, i.e. there was no crossing of the reaction norms. However, a complete crossing of the reaction norms was observed at the intermediate temperature, indicating that the relative fitness of clones can change with food level. Supporting our hypothesis 1, the utilization of low food quantity was more efficient by the Tinaja clone than by the Cueva Morenilla clone, and the utilization of high food quantity was more efficient by the Cueva Morenilla clone than by the Tinaja clone. These findings evidence a performance trade-off between the clones (populations) along the food availability gradient. Moreover, according to the 'threshold hypothesis' (Lampert, 1977; Lampert and Schober, 1980), the best-performing clone in an environment limited by food quantity is the one with the lowest TFL. In our study, the TFL was higher for the Cueva Morenilla clone than for the Tinaja clone at 15.6°C, supporting the greater efficiency of the Tinaja clone in exploiting low food levels. The TFL and r_m values of *K. cochlearis* found in this study are consistent with the observations of Stemberger and Gilbert (1985), who reported an r_{max} of 0.28 day^{-1} and TFL of 0.03 mg C l^{-1} (assuming 50% carbon content of food algae by dry mass) for this species at 20°C when fed on *Rhodomonas minuta*.

A single clone from each population was randomly selected from among the clones maintained in our lab; therefore, there may be a potential selection bias, because clones unable to cope with the laboratory conditions might not have survived. Nevertheless, and despite the close geographic proximity of these populations, our results suggest possible local adaptation to food quantity. Genetic analysis of the two populations showed that they belonged to the same GMYC and ABGD group. The absence of COI sequence divergence suggests that the possible adaptation to local conditions might have occurred after a single historical colonization by the same haplotype (monopolization hypothesis) (De Meester et al., 2002), with the immigration rates of other haplotypes being highly restricted or absent (Declerck and Papakostas, 2017). Sexual reproduction of *K. cochlearis* does not appear to be important in Ruidera Lakes, where low or no production of resting eggs has been observed (Moreno et al., 2017), and no resting eggs were detected in our cultures of this species. Thus, although the frequent waterflow connections between the Ruidera lakes may favor homogenization of the zooplankton communities (Moreno, 2017), the past and

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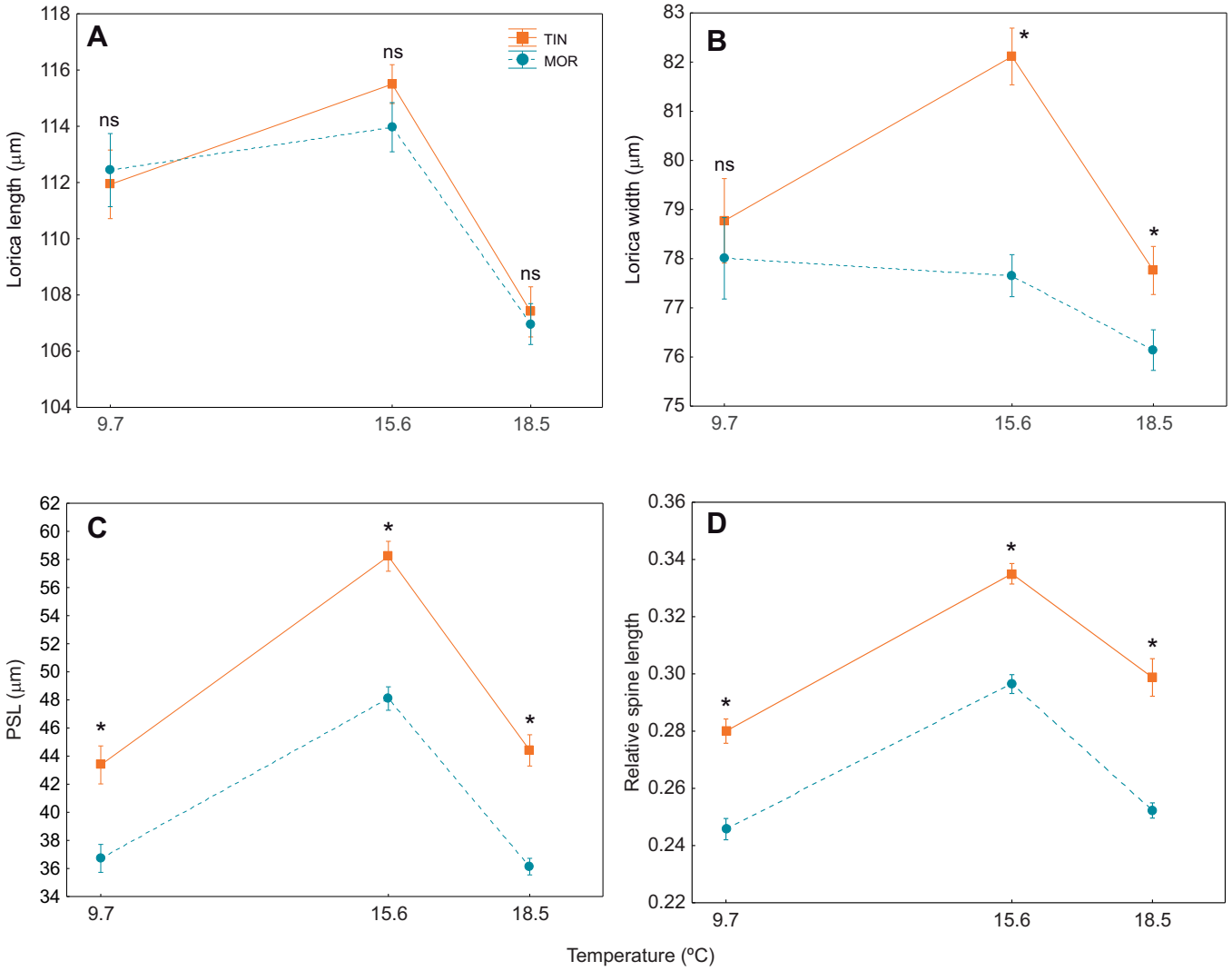

Fig. 4. Thermal reaction norms of morphometrical traits of two *K. cochlearis* clones. Data for (A) length and (B) width of lorica, (C) length of posterior spine (PSL) and (D) relative spine length (PSL:total length ratio) of Tinaja lake (TIN) and Cueva Morenilla lake (MOR) clones (experiment 2). Values are means ±1 s.e.m. of the 13–24 replicate populations at each treatment. *Difference between means (for each temperature) is significant (Mann–Whitney *U*-tests; *P*=0.05); ns, not significant.

present immigration rates of diapausing eggs, males and/or mictic females of *K. cochlearis* from Tinaja lake to Cueva Morenilla lake are very likely to be highly restricted. Regardless, the comparison of neutral genetic markers using microsatellites is needed to elucidate the genetic diversification of these rotifer populations (e.g. Campillo et al., 2009). Our findings are comparable to the results of COI sequence-based phylogenetic analyses for three neighboring populations of *B. plicatilis* complex in Mexico, which were clustered in the same clade and were locally adapted to salinity (Alcántara-Rodríguez et al., 2012).

Different responses of fitness to temperature

Although the importance for zooplankton of local thermal adaptation to global change is increasingly acknowledged (reviewed by Dam, 2013), this issue has been less studied in relation to the cryptic species complex of rotifers, and only the *B. plicatilis* complex (Campillo et al., 2009, 2011) and *B. calyciflorus* complex (Ma et al., 2010) have been investigated. Our experimental results showed different responses of the population growth rate to temperature, suggesting a potential evolution of the reaction norms (e.g. Angilleta, 2009), i.e. local thermal adaptation in the two *K. cochlearis* populations. However, our experimental set-up did not permit the proper testing of local thermal adaptation because the three temperature treatments were not truly replicated (see Materials and Methods), although, our less conservative crossed analyses showed a significant interaction between clone and temperature. In support of this proposition, the lowest experimental temperature (9.7°C) appeared to be more stressful for the Tinaja versus Cueva Morenilla clone, resulting in a lower r_m. In addition, the optimal growth of the Tinaja clone was observed at around 15°C, consistent with the observation in the sole experimental study on this issue that the growth rate of a spine-fixed clone of this species was maximal at 15°C (Walz, 1983). In contrast, r_m values of the Cueva Morenilla clone did not appreciably increase when the temperature rose from 9.7 to 18.5°C. These results suggest that Cueva Morenilla and Tinaja *K. cochlearis* are thermal generalist and specialist, respectively, at least over the tested range of temperature and food quantity. According to the specialist–generalist trade-off theory, this suggests a thermal specialist–generalist trade-off with the generalist phenotype (Cueva Morenilla

clone) providing tolerance of a wider range of temperatures but a lower performance at the thermal optimum of this species (e.g. Dam, 2013). The annual water temperature range is wider in Cueva Morenilla lake than in Tinaja lake, and the minimum water temperature is also slightly lower in Cueva Morenilla lake (Table 1). This is the first study to contribute evidence of genetic differences in temperature tolerance between two neighboring populations of *K. cochlearis* with location-specific temperature variations. To date, only one study has demonstrated that genetically different haplotypes of *K. cochlearis* also differ in their growth rate and in other demographic parameters (Cieplinski et al., 2018). The authors had investigated differences between two ESUs of *K. cochlearis* at the same temperature (14.5°C). In the present study, we observed a differential effect of temperature on the r_m of two closely related *K. cochlearis* clones belonging to the same haplotype and ESU, although they had different phenotypes. This is important because, as suggested by Cieplinski et al. (2017), temperature-related differences in life history traits within the cryptic species complex of *K. cochlearis* may indicate that previously studied populations attributed with wide temperature tolerance (Herzig, 1983; May, 1983; Andrew and Fitzsimons, 1992; Jersabek and Bolortsetseg, 2010) were composed of different ESUs and/or haplotypes with possibly distinct ecological preferences. Further research is warranted on temperature-induced phenotypic plasticity and genetic adaptation to temperature in more haplotypes of *K. cochlearis* and in other rotifer cryptic species in order to understand the response of cryptic species complexes to global warming.

Morphological variation in *K. cochlearis*

Genetic and environmental controls

The temperature-induced phenotypic plasticity of lorica size (LL and LW) and posterior spine elongation (PSL and RSL) in the *K. cochlearis* clones was evidenced in our laboratory study, and the two clones showed distinct phenotypes. Morphological research since 1900 (Lauterborn, 1900) has shown that spine elongation in *K. cochlearis* is controlled by lorica length, food availability, water temperature and predator kairomones (reviewed in Gilbert, 2017). This is the first study to provide evidence of genetic control over the degree of posterior spine elongation in populations of *K. cochlearis*. The distinct genetic potential of different *B. calyciflorus* clones for spine development is well known (reviewed by Gilbert, 2018), but little attention has been paid to other rotifer species. We describe a good example of the way in which non-genetic and genetic factors may interact to control an ecologically relevant morphological trait of *K. cochlearis*. In comparison to the Cueva Morenilla clone, the Tinaja clone had a longer posterior spine, higher relative spine length and wider lorica, but there was no difference in LL. The mean LL and LW values were in agreement with observations in field specimens of ESU 5 by Cieplinski et al. (2017), although they studied spined and spineless specimens and recorded a higher maximum PSL value (113.1 µm) than in the present study. This discrepancy might be attributable not only to the wider temperature range (cold and warm seasons) than in the present experiment but also to differences in the haplotypes detected. In our experiments, the LL and PSL of both clones were much lower with increasing water temperature from 15.6°C to 18.5°C. Unfortunately, Cieplinski et al. (2017) did not indicate the maximum temperature recorded; therefore, the temperature at which unspined forms of *K. cochlearis* were observed is not known. According to Green's (2005) hypothesis on the origin of forms without posterior spines in *K. cochlearis*, our results are coherent with the hypothesis of true tecta (as the end of a reduction series), because the LL was reduced at higher water temperatures. The higher PSL in their observations may also be attributable to the spine-promoting effects of kairomones, low temperature and low food concentration in rotifers (Stemberger, 1990). Under the controlled conditions of our laboratory study, measurements were recorded in the absence of predator and competitor kairomones and with no food limitation, which would explain the lower PSL observed.

It is well documented that variations in the morphological traits (e.g. body size and spine length) of rotifers have adaptive significance in relation to environmental changes. The morphological differences between the two clone populations in our study are consistent with the general consensus that *K. cochlearis* in lakes with lower food availability (e.g. Tinaja lake) tend to be longer and have longer spines than those living in eutrophic conditions (Hillbricht-Ilkowska, 1972; Diéguez et al., 1998; Green, 2007). The fitness benefit of a longer PSL in the Tinaja clone is likely to be a reduced sinking rate (Stemberger, 1990; Zagarese and Marinone, 1992). In addition, their wider lorica could enhance clearance rates (Bogdan and Gilbert, 1982; Stemberger and Gilbert, 1987; Diéguez et al., 1998), because rotifers rotate cilia in the corona to direct food into the mouth (Glime, 2017). Both traits would allow *K. cochlearis* to persist at lower food levels, as observed in Tinaja lake. However, the reduced PSL of *K. cochlearis* in Cueva Morenilla lake might be adaptive for avoiding predation by large vertebrates (larval fish; see Zhang et al., 2017a,b). Although our experiments were carried out in the absence of fish kairomones, the higher pelagic fish biomass observed in Cueva Morenilla versus Tinaja lake (Table 1) may have selected shorter spines in *K. cochlearis* from the former. This change may persist under laboratory conditions because rotifers are known to evolve stable morphological features after long-term local adaptation to their environment (Xue et al., 2017).

Fitness cost of spine elongation in *K. cochlearis*

The development and elongation of spines by rotifers probably involves some energetic cost (reviewed by Gilbert, 2013; Riessen and Gilbert, 2019), explaining why they are only observed when selective pressures are relatively intense, being otherwise disadvantageous (Gilbert, 2013). When food availability is low, spine elongation offers rotifers an adaptive advantage that may reduce their sinking rate and, therefore, swimming costs in the water column (*K. tropica*: Zagarese and Marinone, 1992; *B. calyciflorus*: Stemberger, 1990). Similar effects could be expected for *K. cochlearis*, and the cost of spine elongation would make the Tinaja clone less fit than the Cueva Morenilla clone under conditions of high food availability. Our morphometric analyses of *K. cochlearis* were only performed at a moderate food density (0.5 mg C l^{-1} of *C. pyrenoidifera*), and we cannot draw conclusions on the impact of food density on their spine elongation. In other studies, the right posterior spine of *K. tropica* was found to lengthen with a reduction in food concentration (Zagarese and Marinone, 1992). Hence, lower food densities than used in our study may have a more pronounced effect on the PSL. At any rate, we found no substantial differences in fitness between the *K. cochlearis* clones at a high food concentration at any temperature. The wider lorica in the long-spined rotifers from the Tinaja clone may produce a greater clearance rate (Stemberger and Gilbert, 1987) in comparison to the short-spined rotifers from the Cueva Morenilla clone, favoring their growth rate. Therefore, the possible energetic cost of spine elongation in Tinaja *K. cochlearis* may be less than the fitness benefit of wider lorica. Nevertheless, there is a need for further empirical studies on the costs of plasticity in the *K. cochlearis* complex.

Effect of temperature alone on morphological variation

Our results showed that temperature is a decisive determinant of lorica size and posterior spine elongation in *K. cochlearis*. This is the first experimental study on the effects of temperature alone on the adaptive morphometrical traits of this rotifer, controlling any other environmental signal that can also induce phenotypic plasticity. As expected in ectotherms, *K. cochlearis* had the shortest lorica at the highest tested temperature (107.14±0.56 µm, mean±s.e.m.), consistent with the TSR (Atkinson, 1994). Experimental evidence of an adaptive role for body size in relation to temperature has been documented in other rotifer species such as *Synchaeta pectinata* (Stelzer, 2002), *B. calyciflorus* (Sun and Niu, 2012), *B. plicatilis* (Walczyńska and Serra, 2014) and *Lecane inermis* (Walczyńska et al., 2015). The relationship of LL with temperature found for *K. cochlearis* in the present study is also consistent with the results of numerous seasonal studies of natural populations of *K. cochlearis*, which found an appreciable negative correlation between LL (and PSL) and water temperature (e.g. Bielañska-Grajner, 1995; Green, 2005). Interestingly, however, the lorica size was similar between 9.7°C (112.19±0.36 µm, mean±s.d.) and 15.6°C (114.73±1.08 µm), and this absence of a size reduction with lower temperature may because 10°C is below the optimal thermal range for *K. cochlearis* (Atkinson et al., 2003; Walczyńska et al., 2016). A smaller size can evidently result from stressfully high or low temperatures or from insufficient resources (Atkinson, 1994), consistent with the low r_m of *K. cochlearis* at 9.7°C in our study. The body size of *L. inermis* was also found to be lower at 10°C than at 15°C due to cold stress (Walczyńska et al., 2016).

In addition, LL was more responsive to temperature in comparison to LW. Kiełbasa et al. (2014) observed a similar response in LL of *L. inermis* and also found that LW of *L. inermis* was related to nutritional level; therefore, LW of *K. cochlearis* may respond to food local conditions in their habitats, although this needs to be tested. According to our less conservative analyses (crossed statistical analyses with temperature), the response of LL of both *K. cochlearis* clones was similar across the temperature gradient, although the response of LW differed between them, with a lower response to temperature in the Cueva Morenilla clone than in the Tinaja clone. However, this last result should be considered with caution because of pseudoreplication, as commented above.

An inverse relationship between temperature and PSL has long been reported in *K. cochlearis* from various lakes and ponds (e.g. Eloranta, 1982; Conde-Porcuna et al., 1993; Bielañska-Grajner, 1995; Green, 2005). In the present study, however, the shortest posterior spines were observed at the lowest or highest temperature, while the PSL and RSL were highest at the intermediate temperature, when the posterior spine of *K. cochlearis* was therefore proportionally longer in relation to the lorica size. This result suggests for the first time that intermediate temperatures could activate the gene responsible for spine elongation in *K. cochlearis*. This proposition is consistent with the report by Gilbert (2011) of no change in the length of the right or left spines of *K. tropica* when the temperature rose from 10 to 15°C at high food concentrations (fig. 3 in Gilbert, 2011), although he did not discuss this finding. The posterior spine induced by kairomones of *Asplanchna* in *Brachionus havanaensis* was found to be longer at 20°C than at either 15 or 25°C (fig. 1 in Pavón-Meza et al., 2007). However, this may have been related to a possible change in the metabolic rate of predators resulting from the release of different concentrations of kairomones at this temperature (Riessen and Gilbert, 2019). Unfortunately, Pavón-Meza et al. (2007) did not discuss the relationship between the PSL of *B. havanaensis* and temperature. In our study, we excluded any possible interference from predators on the relationship between temperature and the PSL in *K. cochlearis*. The results obtained indicate that seasonal changes in water temperature would be highly relevant for the morphology of *K. cochlearis*, whose posterior spine is elongated at mid-season water temperatures, coinciding with the usual maximum abundance of *K. cochlearis* in nature (Bērzinš and Pejler, 1989; Vasconcelos, 1990; Yiğit, 2002). No previous study has observed this deviation from the expected negative relationship between PSL and temperature, except for the observation by Carlin (1943) in the 1940s of longer spines in *K. cochlearis* during the spring peak despite the subsequent increase in temperatures. We have no data on the response of our *K. cochlearis* clones to kairomones emanating from their predators; however, the intriguing response of spine elongation to the heating of water from 9.7°C (typical winter) to 15.6°C (typical spring) may correlate with an increased predation risk, and rotifers may use the water temperature as a proxy signaling change in predation risk before defense is needed. The results of laboratory experiments are consistent with the use of water temperature as a proxy cue for predation risk perception in various cladoceran species, inducing the development of defensive structures (helmets, crests, spines, etc.) in warmer waters (Brooks, 1946; Yurista, 2000; Miehls et al., 2013). However, no experimental evidence has been published on this proposition in rotifers. Indeed, the possibility that water temperature could serve as an indirect cue for increased predation pressure was recently described as unlikely (Riessen and Gilbert, 2019). This is because a relationship has been observed between low water temperatures (when predator populations are less abundant or active) and elongated spines in numerous natural populations of rotifers (reviewed by Gilbert, 2017). The present study contributes experimental evidence of posterior spine elongation in the common freshwater rotifer *K. cochlearis* at the moderate temperature of 15.6°C. Further studies are warranted to determine whether this abiotic factor could serve as a signal of predation risk for rotifers in the absence of kairomones.

Conclusions

This study of two populations of *Keratella cochlearis* isolated from neighboring habitats found that: (1) there is intraspecific variation between the populations to food availability, with substantial differences between their phenotypes; (2) both populations show signs of possible local adaptation to food conditions; (3) both populations also show evolutionary potential for local thermal adaptation related to location-specific temperature variations; (4) the degree of posterior spine elongation in *K. cochlearis* demonstrates adaptive phenotypic plasticity; (5) the lorica size of *K. cochlearis* follows the temperature–size rule, and (6) intermediate temperatures favor an increase in PSL compared with more extreme temperatures. To the best of our knowledge, this is the first report of possible local adaptation by *K. cochlearis* to habitats that consistently differ in food availability. This study also contributes relevant morphological and ecological information on the response of a putative ESU of the *K. cochlearis* complex (ESU 5 according to Cieplinski et al., 2017). Our laboratory study findings support the proposition that *K. cochlearis* may use temperature as a proxy cue for predation risk perception in nature, raising new questions about inducible defensive plasticity in rotifers.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: E.R.-R., J.M.C.-P.; Methodology: E.R.-R., E.M., J.M.C.-P.; Validation: E.R.-R., J.M.C.-P.; Formal analysis: E.R.-R., J.M.C.-P.; Investigation: E.R.-R., E.M., J.M.C.-P.; Resources: E.R.-R., J.M.C.-P.; Writing - original draft: E.R.-R., E.M.; Writing - review & editing: E.R.-R., E.M., J.M.C.-P.; Visualization: E.R.-R.; Supervision: J.M.C.-P.; Project administration: J.M.C.-P.; Funding acquisition: J.M.C.-P.

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Data availability
All sequences obtained in this study were submitted to GenBank with accession numbers: MT103157, MT103158, MT103159 and MT103160.

Supplementary information
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