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First record of *Triops gadensis* Korn and García-de-Lomas, 2010 (Crustacea: Notostraca) in Córdoba Province, southern Spain

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SUMMARY

We studied a *Triops* population from a Mediterranean temporary pond located in Guadalcázar, a township in the western part of Córdoba Province. There are four morphologically similar species of *Triops* occurring in the SW Iberian Peninsula. In particular, the two species found in southern Andalusia, *T. baeticus* and *T. gadensis*, cannot reliably be told apart based on known morphological characters. We thus used sequences of 12S rDNA as a molecular marker to soundly identify the population to species rank. Our results demonstrate that the *Triops* population studied belongs to *T. gadensis*. This result is rather unexpected given that the species was thought to be restricted to a small distribution range along the Atlantic coast in Cádiz Province. The population is located at approximately 170 km from previously known sites of *T. gadensis* and shows a previously unknown haplotype, the sixth 12S haplotype reported for the species. We thus suggest that the population has high conservation value, and its habitat, including the surroundings, should be legally protected. We further propose that an open habitat type as pastureland should be retained for the surroundings of the site in order to support its ecological function in the frame of meta-populations dynamics, and as a possible source population from which new localities could be populated via passive dispersal.

INTRODUCTION

The species in the two extant genera of Notostraca, *Triops* and *Lepidurus* are well known for posing particular difficulties to taxonomists. On the one hand, their gross morphology has changed only very little since the Triassic (with the possible exception of the presence/absence of elongated endites in the first trunk limbs, see discussion in Korn et al. 2013). On the other hand, single morphological key characters typically vary to an extent that makes the identification of species boundaries particularly difficult (Longhurst 1955). For example, the number of body segments may vary by up to three in one gender within a single population (Longhurst 1955), and segment boundaries may be blurred by fusion of several "body rings" (a term introduced by Linder 1952 in order to account for the fact that the exoskeleton in most of the body segments is incomplete ventrally, where the row of legs together forms an entity that grows independently from the segments – a most unique case in zoology; Linder 1952, Longhurst 1955). It is thus not surprising that recent molecular studies revealed fundamental discrepancies between the taxonomic classification that was largely accepted for many decades and actual diversification patterns as inferred by DNA sequence data (e.g., Korn et al. 2013).

Regarding *Triops* populations in the SW Iberian Peninsula, recent studies revealed that they do not belong to the Western Moroccan *T. mauritanicus* Ghigi, 1921 [treated as a subspecies of *T. cancriformis* (Bosc, 1801–1802) for some 50 years, from Longhurst's 1955 revision to its resurrection by Korn et al. 2006], but instead they belong to four separate species that were described in Korn et al. (2010). The most widespread of these is *T. baeticus* Korn, 2010, which was recorded from Andalusia, Extremadura, and Portugal. *Triops gadensis* was reported from several localities along the Atlantic cost of Cádiz Province. The remaining two species appear to have even smaller ranges, with *T. vicentinus* Korn, Machado, Cristo and Cancela da Fonseca, 2010 occurring in southernmost Portugal, from western to central Algarve, and *T. emeritensis* Korn and Pérez-Bote, 2010 being endemic to a particularly small range within the Extremadura. Accordingly, the first three species are categorised as endangered at the global level, and the latter species as critically endangered by the IUCN Red List of threatened species (https://www.iucnredlist.org, last accessed 06/01/2024) (see also Boieiro et al. 2023). A first account of the species' status of threat had been provided by García-de-Lomas et al. (2017). In particular, the two most widespread species, *T. baeticus* and *T. gadensis*, are morphologically so similar that a reliable morphological determination is not possible, at least based on known morphological key characters. This makes updates on the biogeography of the species involved particularly difficult as these necessarily rely on DNA sequence-based determinations. In this study, we present the first data from a *Triops* population in Córdoba Province whose taxonomic identity was determined via DNA sequencing.

MATERIALS AND METHODS

The sampling locality is a pond of 65 m^2, located in Córdoba Province in the district Las Colonias within the township Guadalcázar, in a place known as La Dehesilla, at an altitude of 180 m a.s.l. (30SUG27 in the 10x10 km UTM grid) (Fig. 1). Exact coordinates are available from the authors on request. The pond was created for livestock watering and typically fills each year (i.e., it is a seasonal pond *sensu* Grillas et al. 2004). The site is located within a countryside landscape dedicated to agriculture and livestock farming. Historically, the original forests have been cleared since the 14th century (López-Martínez et al. 2022). In the mid-20th century, scattered holm oaks (*Quercus ilex* subsp. *ballota*) and wild olive trees (*Olea europaea* var. *sylvestris*) were still present, forming a landscape locally known as dehesa ("La Dehesilla" means "small dehesa"). The area has

a typically Mediterranean climate (Csa according to Köppen climate classification). The average (of the years 1981-2010) annual temperature and rainfall in Córdoba station, located at approximately 20 km from the pond, is 18 ºC and 605 mm, respectively (AEMET 2023). It presents a continentality index, Ic (average temperature of the warmest month - average temperature of the coldest month) of eucontinental type (Ic = 33.3ºC). There is an average of 15 days of frost per year. Depending on the rainfall regime, the pond fills very irregularly during autumn or springtime. It represents a priority habitat under the EU Habitats Directive (3170* Mediterranean temporary ponds) (Camacho et al. 2009).

Analysis of aerial orthophotographs series from 1956 to 2020 (www.geamap.com) revealed that the pond appears to have been in existence in its present form in 1998 but may have been absent in 1978 (the respective aerial image suggests the presence of a shallow depression), suggesting that it was possibly created (or deepened) about 25 years ago. Orthophotos also revealed a complex of temporary ponds: three additional ponds surround the pond studied (in less than 250 m distance). Two of them are situated in a field that is presently used for the production of agricultural crops. In addition, there is a spring with a fountain 450 m from the pond studied, that persists nowadays.

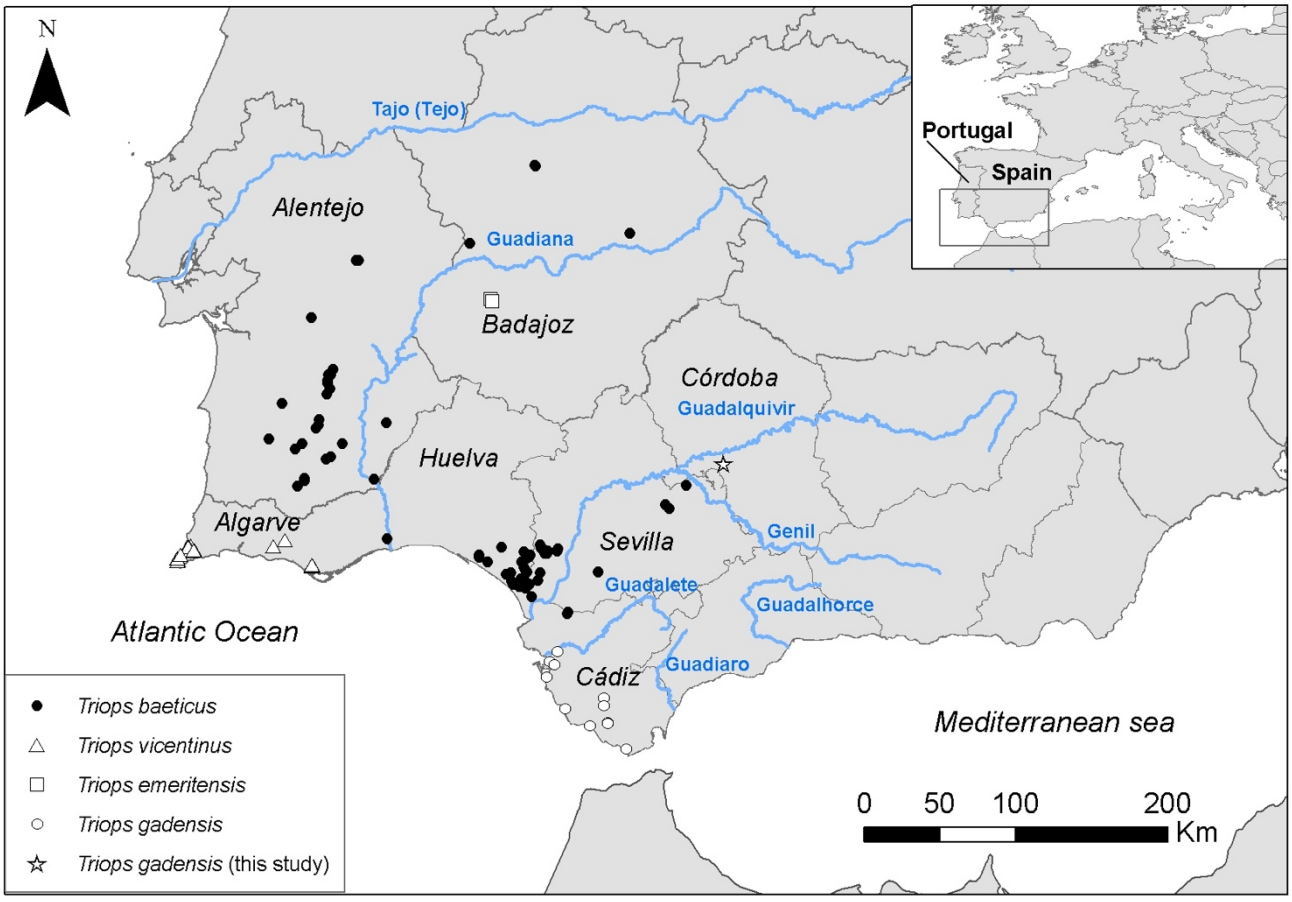

Figure 1. Distribution of *Triops* species in the southwest of the Iberian Peninsula, indicating the new record of *Triops gadensis*. Only populations that were determined via DNA sequencing are shown. The main rivers are indicated in blue.

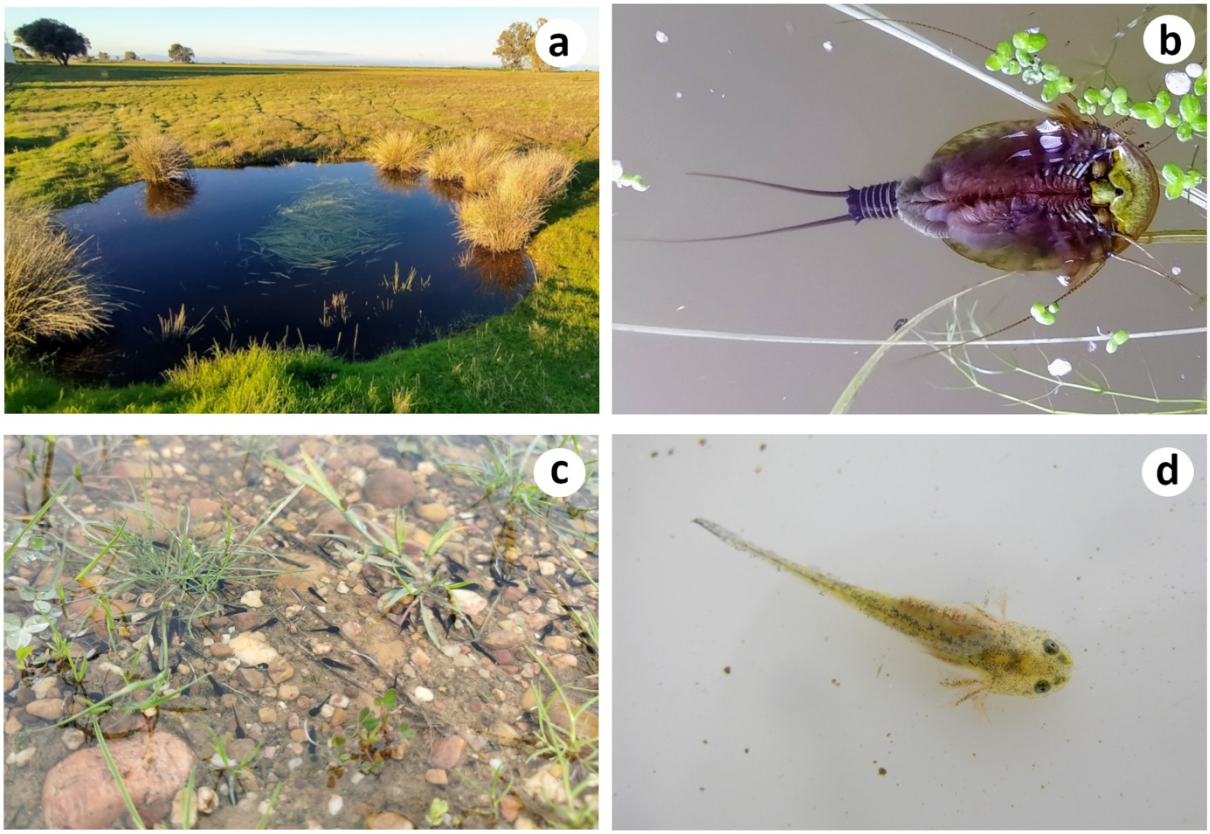

Figure 2. Pictures showing the temporary pond studied, as seen in February 2023 (a) and examples of its fauna, including the tadpole shrimp *Triops gadensis*, newly recorded in Córdoba province (b), and larval stages of two protected amphibian species: the natterjack toad (*Epidalea calamita*) (c), and the Iberian ribbed newt (*Pleurodeles waltl*) (d). Photos R. Pulido (a-c) and J.M. Delgado (d).

Despite its recent, man-made origin, the pond studied has developed a biocoenosis typical of mature temporary ponds (Fig. 2): aquatic macrophytes and helophytes include *Glyceria declinata* Brébisson, 1859, *Callitriche lusitanica* Schotsman, 1961, *Damasonium alisma* Miller, 1768, *Damasonium bourgaei* Coss, 1849 and *Scirpoides holoschoenus* (Linné) Soják, 1972. It is inhabited by three protected species of Amphibia, *Pleurodeles waltl* Michahelles, 1830, *Epidalea calamita* (Laurenti, 1768) and *Pelodytes ibericus* Sánchez-Herráiz, Barbadillo, Machordom & Sanchiz, 2000. The crustacean fauna includes the large branchiopods *Triops gadensis* and *Chirocephalus diaphanus* Prévost, 1803, and the copepod *Mixodiaptomus incrassatus* (Sars, 1903). Unfortunately, no other data about the co-occurring crustacean fauna are to date available.

Triops specimens were sampled in February 2023, after rains in the winter of 2022-2023 had filled the pond (253.24 mm precipitation recorded from December to February) (www.tutiempo.net/clima/ws-84100.html, last accessed 18/01/2024). A representative sample of the specimens collected is stored at the University of Granada [Nº Cat. 22475-CG (CCZ-UGR)]. Two individuals were randomly chosen for a DNA-based species identification of the population. We used a fragment of the 12S rDNA since this marker has been studied in detail in Andalusian populations (Korn et al. 2010), so that a rich comparative dataset is available; 12S rDNA has often been used for the identification of *Triops* species based on single marker sequences (e.g., Tziortzis et al. 2014; Marrone et al. 2019). Used primers, PCR mix, and thermal cycles followed Korn et

al. (2006, 2010), but PCR products were sent to Macrogen Europe for sequencing. We used comparative sequences of 12S rDNA available for *Triops mauritanicus*, *T. baeticus*, *T. gadensis*, *T. vicentinus* and *T. emeritensis* (henceforth collectively referred to as *Triops mauritanicus* morphogroup), as well as *T. simplex* Ghigi, 1921. Two sequences of *T. cancriformis* served as outgroup. GenBank Accession numbers are shown in Figure 3. Sequences were aligned using Clustal W (Thompson et al. 1994), as implemented in BioEdit version 7.2.6 (Hall 1999). A Maximum likelihood (ML) analysis was performed in RAxML-NG (Kozlov et al. 2019). Applied settings were 'ML + thorough Bootstrap + consensus', 100 runs and 1000 bootstrap replicates. The graphical user interface raxmlGUI 2.0 (Edler et al. 2021) was used for executing the program. The best evolutionary model was inferred by ModelTest-NG (Darriba et al. 2019) as implemented in raxmlGUI 2.0. The selected model was TPM2uf + G4.

The area of occupancy (AOO) was calculated using the GEOCAT tool (http://geocat.kew.org/, last accessed 06/01/2024). according to IUCN standards (2012).

RESULTS

The 12S rDNA sequences obtained indicate that the new population belongs to *Triops gadensis* (Fig. 3). Both sequenced individuals show a new shared haplotype that is differentiated from previously known 12S haplotypes of *T. gadensis* by mutations in 2 to 7 base-pairs, corresponding to 0.37–1.28% pairwise sequence divergence (uncorrected p-distances). The new haplotype has been uploaded to GenBank with the Accession Number PP256049. All previously DNA-determined populations within the valley of the Guadalquivir River belong to *T. baeticus* (see Fig. 1; Fig. 3 in Korn et al. 2010). Both species were already known to show a parapatric distribution (Korn et al. 2010), and the new record of *T. gadensis* shows a geographic distance to *T. baeticus* that is only slightly higher than the previously reported minimum geographic distance of 25.8 km observed between both species (Korn et al. 2010): the population of *T. baeticus* reported for Cañada Rosal, Sevilla Province (population no. 32 in Table A1 in: Korn et al. 2010) is located at approx. 28 km from the population studied herein.

DISCUSSION

The population studied does not represent the first record of a population of the *Triops mauritanicus* morphogroup in Córdoba Province. Alonso (1985) reported it at Benamejí (cited as *T. cancriformis mauritanicus*; the second site reported in the same study, 'La Miaha', actually refers to a locality in Sevilla Province: M. Alonso, University of Barcelona, pers. comm.). More recently, Prunier and Saldaña (2010) detected it in nine out of the 113 temporary ponds they searched for large branchiopods throughout Córdoba Province, including two ponds at La Dehesilla. Prunier et al. (2011) reported *Triops* from 12 ponds, four of which appear to refer to previously known sites in Prunier and Saldaña (2010). Prunier et al. (2011) referred to the populations as *Triops* sp., but since they suggest that the samples likely refer to *T. baeticus* (based on its known range), it appears clear that these samples also conform to the *T. mauritanicus* morphogroup. Unfortunately, it is presently not possible to infer to which of the morphologically very similar species of the *T. mauritanicus* morphogroup these previous records belong to. Since the population we studied occurs in an excavated pond, we assume that it is the result of a recent dispersal event. It shows a haplotype in 12S rDNA that had not been previously reported despite a rather high number of individuals for which DNA sequences are available: 12S sequences had been obtained from a total of 55 individuals of *T. gadensis*, as can be inferred from Table A1 in Korn et al. (2010). We thus assume that the pond at La Dehesilla has not

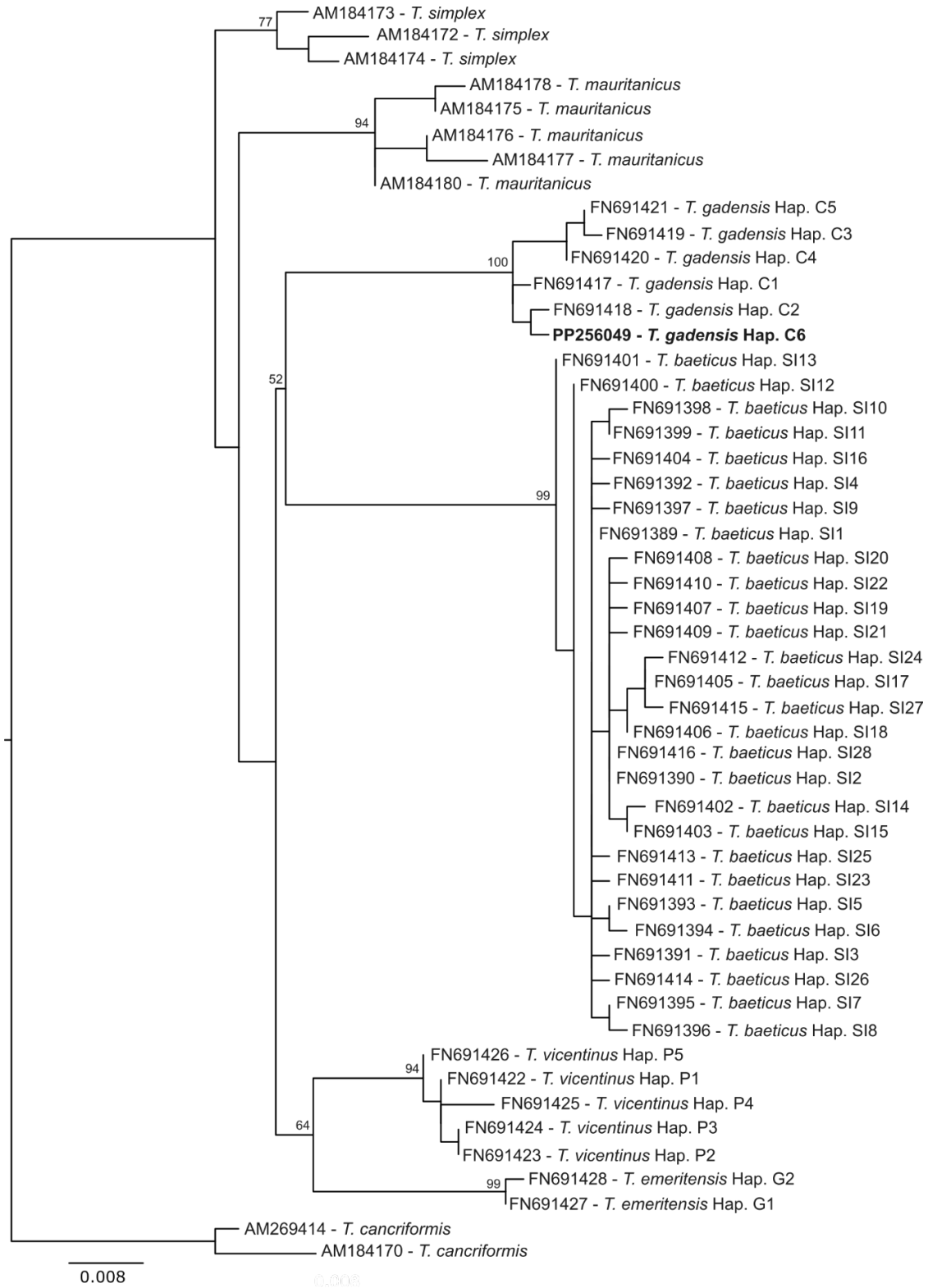

Figure 3. Phylogenetic relationships of *Triops* samples belonging to the *T. mauritanicus* morphogroup and *T. simplex*, as inferred from 12S sequences. (The best scoring tree obtained in RAxML-NG is shown). The tree is rooted on *T. cancriformis*. The haplotype found in the *Triops* population from the pond studied here is written in bold. The GenBank entries use haplotype names that are based on the provisional taxon identifiers of Korn et al. (2010). C: Cádiz; SI: S. Iberia; P: Portugal; G: Gitanilla.

been populated by *T. gadensis* via dispersal from its previously known range, but instead from another, possibly nearby pond. Wild mammals or livestock could have been involved as dispersal vectors in this colonisation process (see e.g., Thiéry 1987, Korn et al. 2010). However, birds are likely to have also been involved in the dispersal to the region where La Dehesilla is located, because it must be regarded as being separated from the populations in Cádiz Province by two rivers, if dispersal across mountainous regions is considered unlikely (compare Fig. 1 to Fig. 3 in Korn et al. 2010). The records of *Triops gadensis* that were previously known are located south of the Guadalete River, whereas the known sites of *Triops baeticus* are all located north of the Guadalete River. The pond studied here is separated from *Triops baeticus* populations by the Genil River (Fig. 1). Further studies on *Triops* species' distribution in presently understudied regions of the Southern Iberian Peninsula, in particular the corridor to the east of the Guadalete and Genil rivers, could shed more light on the dispersal and differentiation processes associated with the *T. mauritanicus* morphogroup.

It is noteworthy that the known geographical ranges of the four Iberian species of the *Triops mauritanicus* morphogroup are in part close together, but they do not appear to overlap with each other (see Fig. 1; Fig. 3 in Korn et al. 2010). This appears to suggest that range expansions of any of the species into regions already occupied by a different one of these species is usually not possible, i.e., the species appear to reciprocally act like dispersal barriers to each other (Korn et al. 2010). This appears to apply also to *T. cancriformis*, which was found to occur at the northern range boundary of *T. baeticus* in Portugal (Machado et al. 2017a, b).

Since all previously DNA-determined populations within the valley of the Guadalquivir River were found to belong to *T. baeticus*, we would have expected that also the population studied herein would belong to this species. Its identity as *T. gadensis*, in a locality at approximately 170 km distance to any other known population of the species, was unexpected given that the species seemed to be restricted to the lowlands along the Atlantic coast of Cádiz Province. We would have assumed that *T. baeticus* would have hindered a range expansion of *T. gadensis*. This would suggest a possible involvement of a long-distance dispersal event by birds. However, it also has to be considered that conditions for a range expansion via repeated short-distance dispersal could have been more favourable in the past. For example, the closely located populations of *T. baeticus* at Cañada Rosal and Fuentes de Andalucía (Korn et al. 2010), that may act like a barrier to dispersal (see above), could be the result of a rather recent range expansion from the Guadalquivir River delta, following deforestation. As was pointed out by Korn et al. (2010), phases where open landscapes were dominating are likely to have favoured dispersal. This is also in good accordance with the evidence that the realized dispersal in large branchiopods is higher in arid areas and open grassland than in areas characterized by higher habitat complexity due to vegetation structure surrounding the ponds (see Stoch et al. 2016).

The population we studied shows a 12S haplotype that had not been recorded within the previously known range of the species. Because of this, and due to the assumed recent origin of the habitat (see above), we think that our study pond likely was populated from another, possibly nearby site (i.e., a pond with a *Triops* population that to date has not been included in molecular studies).

As mentioned above, several occurrence sites of the *T. mauritanicus* morphogroup were recently reported by Prunier and Saldaña (2010) and Prunier et al. (2011). One of these populations, that to date could not be determined to species, could represent the origin of the population we studied. We had ourselves identified a possible source population in a closely located large natural temporary pond in

2010 (M. López-Martínez, pers. obs.). Unfortunately, this population, which clearly belongs to the *T. mauritanicus* morphogroup, was found in the course of a general survey of the flora and fauna of the region, and taxa were not identified at species level. No *Triops* specimens have thus been preserved for DNA identification at that time so that we were not able to determine it to species. The pond in question is also located at La Dehesilla, at 1.7 km to the pond studied herein (within the same square 30SUG27 in the 10x10 km UTM grid). It likely corresponds to one of the two sites of La Dehesilla studied by Prunier and Saldaña (2010). These authors suggested that the temporary ponds at La Dehesilla are among the most important large branchiopod sites in Córdoba Province. We suggest that further, detailed investigation of these biotopes is urgent and desirable. Our observations of this possible source population were made after outstanding heavy rains in the winter of 2009-2010 (967 mm) had filled the pond. Such outstanding rainfall had only been previously recorded in 1976, with 933 mm, and in 1996, with 951 mm (http://www.tutiempo.net/clima/ws-84100.html, last accessed 06/01/2024). The pond has not been flooded any more since then. Such occasional flooding is per se typical of episodic ponds (*sensu* Grillas et al. 2004). However, the occurrence of intensely cultivated olive groves nearby, withdrawal of groundwater and even geologic movements may have altered the flooding regime of the pond. We suggest that this pond should be protected although it is not clear if it is still intact. Observations on laboratory cultures suggest that the recently observed prolonged dry phase may already have lasted for too long time in order to retain viability of the resting stages of *Triops* in this pond (M.K., pers. obs.). It is thus even more important to take measures of protection of the small man-made pond that we studied for which we have evidence that it supports a viable *Triops* population.

In the absence of further data, we must consider the possibility that the *Triops* population we studied could represent the single extant population of *T. gadensis* outside Cádiz Province. This additional locality slightly increases the area of occupancy of the species, from 44 km^2 (García-de-Lomas et al. 2017) to 48 km^2. However, according to IUCN Red List criteria (IUCN, 2012), the category of threat remains unaffected by that, i.e., the species still must be classified as "endangered" at a global scale. In particular, in the face of ongoing climate change, the preservation of this isolated population may be of particular importance: local climatic conditions may differ from those found within the main range of *Triops gadensis*, so that its persistence may become relevant for the long-term survival of the species. Genetic diversity may be a crucial factor with respect to the ability of the species to adapt to rapidly changing environmental conditions. *Triops gadensis* shows rather low genetic diversity, as inferred from the number of 12S rDNA haplotypes. Including the new haplotype that we detected in the pond at La Dehesilla, a total of six 12S haplotypes are known for the species, as compared to 28 haplotypes reported for *T. baeticus* (see Fig. 3; Fig. 2a in Korn et al. 2010). This may generally make *T. gadensis* more prone to extinction.

Our results add conservation value to the site at La Dehesilla: first, *Triops gadensis* is endemic to the Iberian Peninsula, only present in southern Andalusia, and currently considered endangered at the global level (IUCN 2024); second, *Triops* spp. are flagship species of Mediterranean temporary ponds, which are habitats of priority community interest according to Directive 92/43/EEC and Spanish Law 42/2007 on Natural Heritage and Biodiversity; third, the pond studied is an essential habitat for the reproduction of Iberian ribbed newt (*Pleurodeles waltl*), the natterjack toad (*Epidalea calamita*) and the endemic Iberian parsley frog (*Pelodytes ibericus*), all included in the List of Wild Species under Protection Regime according to Spanish and Andalusian legislation (Royal Decree 139/2011, Decree 23/2012). We thus propose that the pond at La Dehesilla that we studied should be legally

protected, together with a buffer zone of adequate size to sustain key ecological processes. We suggest that the present use of its surroundings as open pastureland should be retained: the possible role of the pond as a source population from which new habitats could be colonised may depend on its attractiveness to waterbirds, which is favoured by open habitats (see Korn et al. 2010, Stoch et al. 2016, and citations therein).

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AUTHOR CONTRIBUTIONS

MLM, FM & MK planned and headed the project. MLM carried out the field work. LV, FPF & MK carried out the laboratory work and the analyses. MK and JGL wrote a first draft of the manuscript, which was discussed and improved by all the authors.

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