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ORIGINAL ARTICLE

Reproduction of *Daphnia pulex* in a Northern Italy pond

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ABSTRACT

This paper reports on the occurrence of Daphnia pulex *in a small fishless water body of Northern Italy (*Bodrio del pastore III*) and describes its population dynamics.* Bodrio *is a typical water body originated by erosion from the old Po river-bed surface. During a three year study (2011-2013),* D. pulex *population showed an increase in density from spring to early summer, it declined in July-August and did not recover, presumably from ephippia, until the following spring. The seasonal dynamics was related to the species thermal tolerance and to invertebrate predation by* Chaoborus *that resulted in juvenile high mortality and in adaptive predator-avoidance cyclomorphosis. Seasonal variation was observed in the frequency of individuals, most juveniles, showing neckteeth.* D. pulex *population reproduces by cyclical parthenogenesis and showed a very early investment in sexual reproduction, independent of population density. Males and ephippial females appeared at the beginning of growth season before the density peak according to a typical feature of* Daphnia *populations from temporary habitats. This suggests a lower influence of the environmental factors on sex determination compared to populations inhabiting more permanent habitats. Lineages that produce males and ephippial females early in the season had lower growth rate than parthenogenetic ones but may preserve from selection pressure by predators a significant percentage of genetic variation linked to sex allocation so that evolution proceeds toward earlier and increased sexual reproduction. Our results suggest further investigation to verify whether the current population is the last remnant of a once much larger metapopulation eventually reduced by anthropogenic disturbances or if it represents the invasion of North American strains.*

Key words: Invertebrate predation, cyclomorphosis, cyclical parthenogenesis, sex allocation, recruitment from ephippia.

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INTRODUCTION

Daphnia pulex Leydig, 1860 is a complex of species studied by limnologists for over a century and no group of freshwater zooplankton has been more intensively studied (Kerfoot, 1980; Peters and de Bernardi, 1987; Colbourne and Hebert, 1996; Seda and Petrusek, 2011).

D. pulex is globally distributed throughout the temperate zone and inhabits remarkably different types of lentic habitats (Colbourne *et al.,* 1998). It plays pivotal roles in aquatic food webs, serving as primary grazers of algae, bacteria, and protozoans, and as primary forage for fish (Tessier *et al*., 2001). It is an important model organism in (developmental) phenotypic plasticity that involves a vast number of traits (*e.g*., size, morphology, aging, behaviour) and can be induced by a variety of environmental cues (*e.g*., temperature, predator kairomones, toxins) (Parejko and Dodson, 1991; Spitze, 1992; Weider and Pijanowska, 1993). In the most recent developments of environmental genomics, the complete sequence of its genome will provide an exceptionally powerful tool for addressing fundamental issues in ecology, ecotoxicology, and evolution and in understanding genome-environment interactions (Eads *et al*., 2008; Schaack, 2008; Colbourne *et al*., 2011).

Most studies of *D. pulex* have focused on North American populations because of its dominance in freshwater habitats. *D. pulex* propensity for hybridization and its variation in reproductive mode provided an excellent opportunity to explore breeding system diversity and ploidy levels along geographical gradients (Adamowicz *et al*., 2002; Jose and Dufresne, 2009; Vergilino *et al*., 2011). Temperate populations reproduce by cyclical parthenogenesis when one or more parthenogenetic (asexual) generations alternate with a sexual generation, generally cued by factors that are indicative of environmental deterioration (*i.e.* low food level, crowding, changes in temperature and photoperiod) (Stross, 1987; Koch *et al*., 2009). Some (asexual) lineages have made permanent transitions to obligate parthenogenesis, some asexual lineages are polyploid and are prevalent in arctic and in some high alpine areas whereas diploid lineages dominate in temperate zones (Innes and Hebert, 1988; Weider *et al*., 1999; Hebert and Finston, 2001; Adamowicz *et al*., 2002; Weider and Hobaek, 2003; Aguilera *et al*., 2007; Mergeay *et al*., 2008). In North America, eastern populations are strictly asexuals, mixed populations (both sexuals and asexuals) are found in Ontario, whereas western populations reproduce by cyclical parthenogenesis (Beaton and Hebert, 1988). The ecological differentiation of clonal lineages suggested that they have diverged physiologically into ecotypes as a result of local adaptations (Weider and Hebert, 1987; Dufresne and Hebert, 1998).

The identity of European and American populations of morphologically similar species may be doubtful (Hrbacek, 1987). The accumulation of genetic data has showed that North American and European *D. pulex* are genetically two distinct species and nomenclature of the *D. pulex* complex is not resolved (Colbourne *et al*., 1998; Palsoon, 2000; Mergeay *et al*., 2006; Petrusek *et al*., 2008; Crease *et al*., 2012; Markova *et al*., 2013). Even in Europe, asexual strains of *D. pulex* show a more northerly distribution than sexual ones (Ward *et al*., 1994; Weider and Hobaek, 1997) and both lineages may co-exist within the same geographical region (Letho and Haag, 2010). In freshwater rock pools on islands of the Baltic sea archipelago (Southern Finland), the asexual strains belong to the North American clade whereas the sexual strains belong to the European clade (Letho and Haag, 2010).

In Italy, the geographical distribution of *D. pulex* is broad but patchy, and probably over-estimated as the status of many populations is quite complicated by the existence of *D. obtusa* Kurz and *D. curvirostris* Eylmann, 1878 (Margaritora, 1985). *D. pulex* was recorded both in permanent and temporary habitats in Northern and Southern Italy (de Bernardi *et al*., 1985; Stoch *et al*., 2002; Marrone *et al*., 2007; Leoni and Garibaldi, 2009) and in several Italian islands: Sicily, Sardinia, Lampedusa and Capraia (Stoch *et al.*, 2002; Marrone *et al*., 2006). By molecular markers, panarctic and North American *D. pulex* individuals were identified in Northern Italy and in Sardinia (Vergilino *et al*., 2011; Markova *et al*., 2013).

In April 2011, during a practice field trip, *D. pulex* was collected in a *bodrio*, located in the Cremona province in Northern Italy and in a nearby ephemeral pond. *Bodrio* is a word derived from the greek *bòthros* which means *hole, pit* and it indicates a typical water body originated by erosion and evorsion from the old Po river-bed surface. In the Cremona province, at least 61 *bodri* have been listed. Major biodiversity and conservation studies on these habitats are based on vertebrates and plants (D'Auria and Zavagno, 1999). To our knowledge invertebrate freshwater groups need to be studied considering ponds as model systems in conservation ecology (De Meester *et al.*, 2005).

The aim of the present paper was to report on the occurrence of *D. pulex* in the *Bodrio del Pastore III* and to describe its seasonal population dynamics and reproductive mode. This is a contribution to the knowledge of *D. pulex* in Europe.

METHODS

The *Bodrio del pastore III* (28 m asl, 45° 00' 05" N 10° 19' 26" E, Hayford ellipsoid) formed presumably between 1723 and 1870 and is located in cultivated lands in Cremona province (AAVV, 1999). It has a surface area of about 3.130 m^2, a perimeter of about 196 m and a maximum depth of 6.30 m. It is considered a permanent water body and its volume is affected by River Po level, aquifer, precipitation, runoff and river inundations.

Periodical surveys were carried out from May 2011 to June 2013 and the bodrio was more intensively sampled in spring and early summer (Fig. 1). Zooplankton was collected by 105 μm-mesh net. From 2 to 16 l of water were filtered for each sample according to the water volume and depth. Each sample was preserved in 90% ethanol. During each survey, water temperature was measured a few centimetres below the surface and maximum depth was recorded. The water volume was estimated by considering the bodrio conical shape according to the formula: $V=(S * h)/3$ where S is the surface area and h is the maximum depth. Meteorological data about daily air temperature and rainfall were from the weather station *LaCrosse WS2350* of Gussola.

Most cladocerans were identified to species level according to Margaritora (1985). Abundance of *Daphnia* was enumerated using 100% of each sample and the number of juveniles, adult males, adult females, ovigerous females and ephippial females was recorded. Body size (body length, from apex of head to the base of the tail spine) and spine length of at least 50 *Daphnia* females per samples were observed and measured using a stereomicroscope Nikon SMZ 2T and the Nis-Elements D 3.1 software (NIKON NIS Elements Imaging Software).

Regression analyses, Shapiro-Wilk test for normality distribution, Mann-Whitney and median non parametric tests were performed using SPSS 21.0 software.

RESULTS

Sampling was prevented by ice-cover in February and December 2012 and by adverse weather in the period January-March 2013. During the study, depth varied between 0.80 and 6.10 m with a mean value of 2.20 m (SD 1.58). Accordingly, the *bodrio* did not dry up. Its volume varied between 200 m^3 (from August to November 2012) and 4321 m^3 (June 5 2013). In spring 2013, both abundant rainfall and Po flood (May 21st, 2012), accounted for its maximum volume. Water temperature recorded on sampling date was related to the mean air temperature recorded during 15 days before sampling date (R^2=0.793; $F_{1,38=}$150.576; P<0.001).

In the zooplankton assemblage 6 taxa of Cladocera were recorded: *Alona* sp., *Ceriodaphnia dubia, Chydorus sphaericus, D. pulex, Moina micrura, Picripleuroxus denticulatus*, *Schapholeberis kingi* and *Simocephalus vetulus.* Most of these species had their maximum development in spring. Only the small species *S. kingi* showed a slight presence in all seasons but winter time. *M. micrura* was recorded only in June 2011. *D. pulex,* as well as *S. vetulus,* showed an increase in density from spring to early summer, declined in July-August and did not recover until successive spring. *Chaoborus flavicans* larvae were found: their density was high and almost constant in 2011 with the exception of the warmest months July and August and decreased significantly in the second half of 2012 and 2013 when only few individuals were recorded only in 3 samples (October 6 2012, April 24 2013 and June 5 2013). Quantitative data on *Chaoborus* density are not available.

In all the years the study was carried out, the *D. pulex* population was re-established in spring, probably from resting eggs (Fig. 2). The summer samples showed an increase in density and peaks were recorded just before a sharp population decrease in June-August, according to the year. In autumn and winter no *Daphnia* was recorded, but only very few juveniles in November 2011. Total density fluctuations were related to water temperature: population density increased during June-July accordingly and peaked at temperature between 15 and 20 °C. Maximum density was observed on June 5th 2013 but population disappeared on June 18th 2013 when water temperature increased up to 29°C (Fig. 2). As expected, total density was related to juveniles density (R^2=0.921; $F_{1,31}$=375.32; P<0.001) that increased in summer due to the parthenogenetic reproduction phase (Fig. 2). Throughout the three year sampling period, juveniles of both gender, adult males, ephippial females and free ephippia were recorded throughout all the sampling period indicating that the sexual phase of cyclical parthenogenesis anticipates the population density peak (Fig. 3). Seasonal variation was observed in the frequency of individuals, most juveniles, showing a neckteeth, small spines on the dorsal part of the carapace that are adaptive predator-avoidance morphological traits (Fig. 4). In general, body size distributions of individuals with neckteeth (NT) and neckteethless ones (NL), were not normal and they were significantly different among years (Mann-Whitney P<0.001) (Fig. 5). In 2011, most *Daphnia* juveniles of both gender showed a neckteeth through June and July months. Accordingly, NT females (n=167, mean 899.8±9.71 µm, median=874.7 µm) were significantly smaller than NL adult females (n=57, mean 1057.4±41.25 µm, median=947.21 µm) (median test, P=0.014). In 2012, most juveniles were NL: body size of NT females (n=45, mean 892.1±23.14 µm, median=879.94 µm) was significantly smaller than that of NL females (n=193, mean 1048.1±28.87 µm, median=958.93 µm) (median test, P=0.047). In 2013, all measured individuals of ex-ephippial generation and through May were NL (n=304, mean 971.75±13.36 µm, median=896.53 µm) (Fig. 5). On June 5th most individuals showed a neckteeth and their body

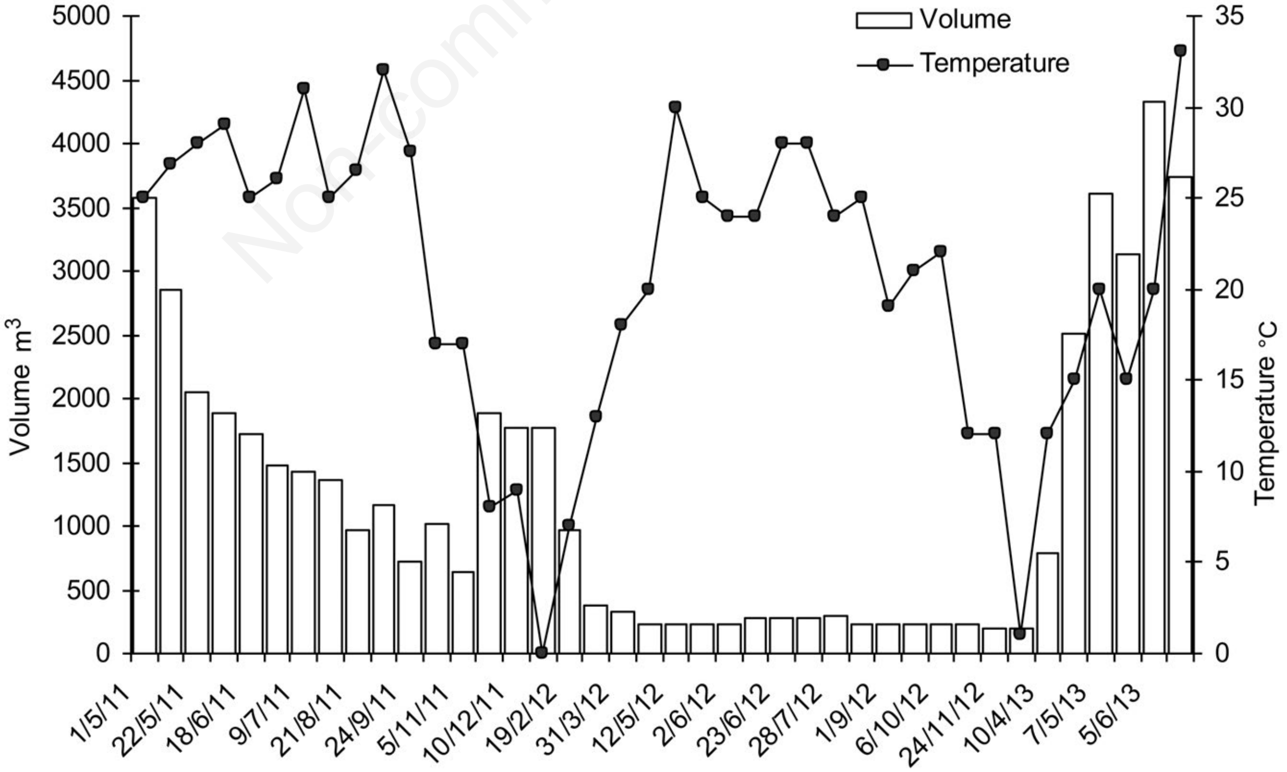

Fig. 1. Seasonal variation of water volume at sampling date and mean of air temperatures recorded during a 15 day period before sampling.

size distribution was normal (n=84, mean 1042.15±9.94 µm, median=1051.24 µm); NL individuals were significantly larger (n=20, mean 1196.53±54.64 µm, median=1147.28 µm) than NT ones (median test, P=0.006). In general, NT juveniles were smaller (median test, P<0.001) and showed smaller body-tail spine ratio (median test, P<0.001) than NL ones. Total length, that is body plus spine size, varied significantly among years in juveniles (median test, P<0.001) but not in adults (median test, P<0.054). Adult total length ranged from 1181 to 2565 µm.

DISCUSSION

Daphnia pulex was present in the *Bodrio del pastore III* only during spring and early summer, it declined and disappeared in late summer and recruited, the following spring, presumably by hatching of resting eggs. Thermal regime and the presence of the predaceous larvae of *Chaoborus flavicans* might account for the observed population dynamics and cyclomorphosis. The presence of active high density population was recorded when temperature was between 15 and 22°C, a tolerance range that is in accordance with general reports on *D. pulex* (Margaritora, 1985; Havel, 1985; Black and Dodson, 1990) and with data from the Italian population of Lago Grande di Monticchio that was described by Leoni and Garibaldi (2009). The presence of *C. flavicans* might account for high juvenile mortality reducing the chance of parthenogenetic females to reach maturity. The highest population density observed in late spring 2013 was probably related both to lower mean temperature and lower predator density than in 2011 and 2012. However these were not the only factors affecting *D. pulex* population dynamics. In fact, in autumn, when temperature could be optimal, the species was not recorded with the single exception of 4 juveniles in November 2011. Assuming that the population recruitment is from ephippia, it may be that, in autumn, due to the so called *refractory phase*, ephippial eggs simply do not hatch (Stross, 1987; Rossi *et al.,* 1998; Clark *et al*., 2012). Although we cannot rule out the effect of other factors (*e.g*., food quantity and/or quality, temperature) and we have no quantitative data on *C. flavicans* density, *Chaoborus* predation may account for seasonal variation of juveniles morphology. It is well known that, in presence of *Chaoborus* larvae, *D. pulex* developed neckteeths and/or elongated tail spines, especially in juvenile individuals, as inducible defences that increase the resistance to invertebrate predation (Havel, 1985; Col-

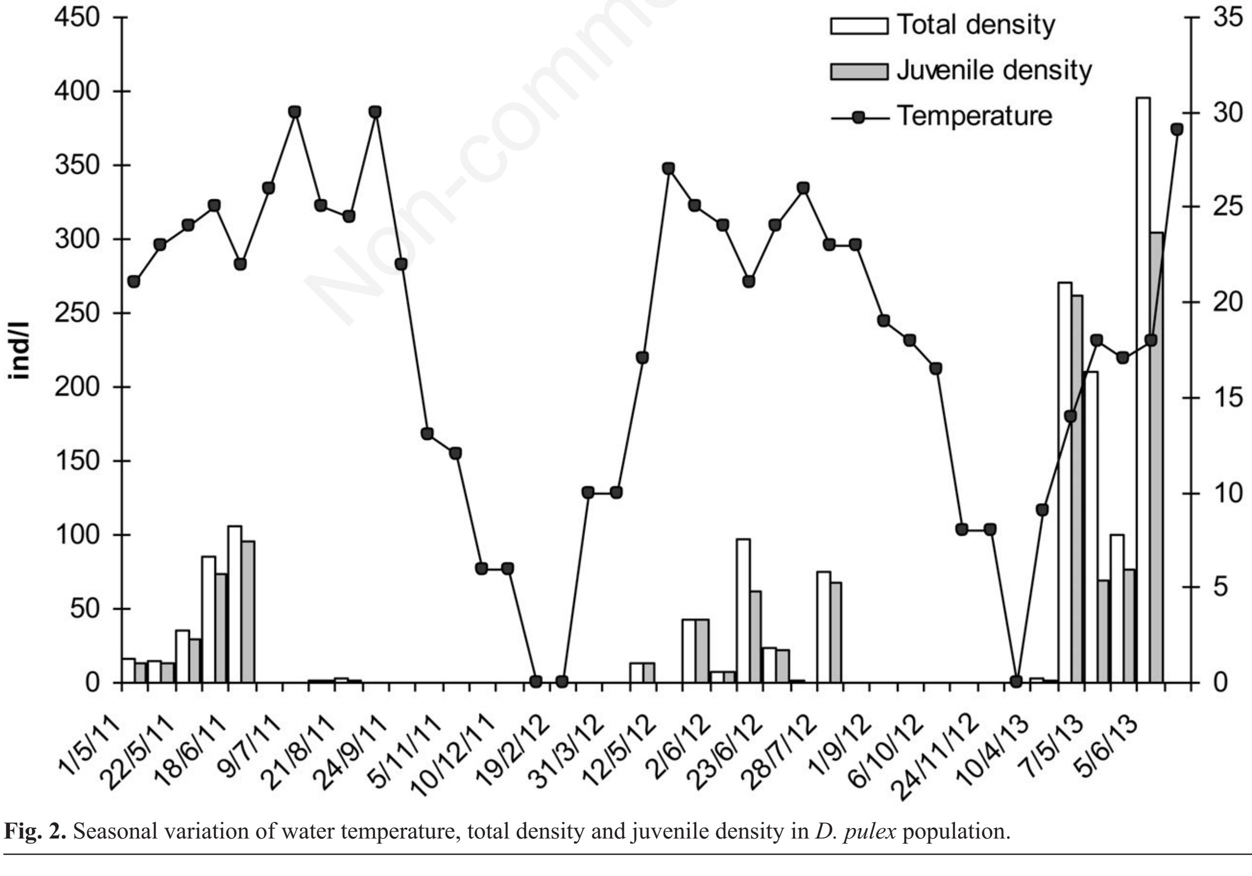

Fig. 2. Seasonal variation of water temperature, total density and juvenile density in *D. pulex* population.

bourne *et al*. 1997; Kotov *et al*., 2006; Spanier *et al*., 2010; Herzog and Laforsch, 2013). According to Havel (1985), the proportion of juveniles which bears neckteeths is a positive function of both temperature (near or above 10°C) and predator density (near or above 1 L^{-1}). Our report is interesting because this phenomenon appears more common in North American than in European populations (Havel and Dodson, 1984; Hrbacek, 1987; Repka *et al*., 1995; Juracka *et al.*, 2011).

The question is whether cyclomorphosis occurs via phenotypic plasticity of one or few clonal lineages that produce different phenotypes under different environmental conditions (*e.g*., kairomone dose and/or temperature). Alternatively, it should be due to clonal succession when the relative frequency of genotypes with different ability to develop a neckteeth changes during the season as a result of positive or negative selection by *Chaoborus* (or other environmental factors). According to Spitze (1991), *Chaoborus* predation removed 93-99% of initial genetic variability in 8-12 generations of clonal reproduction in *D. pulex* inhabiting temporary ponds. Within populations, not all genotypes are likely to respond in the same way to predator stresses and strong interclonal variability is well known (Parejko and Dodson, 1991; Weber and Declerck, 1997), including opposite reactions within one species (Spitze, 1992; Boersma *et al*., 1998; Pauwels *et al*., 2005). There is indication that the production of neckteeths must occur during embryonic development, involve substantial strengthening of the carapace, may have demographic costs and may be poorly adaptive in other ways (Weber and Declerck, 1997; Wiebke *et al*., 2005; Juracka *et al.*, 2011). The production of neckteeths appears to reduce the amount of resources for eggs and females exposed to *Chaoborus* kairomones usually tend to mature later, at larger size, and to produce fewer but larger newborn (Luning, 1992; Spitze, 1991; Riessen, 1992; Spitze, 1992; Tollrian, 1995; Weber and Declerck, 1997; Agrawal *et al*., 1999; Riessen, 1999; Boeing, 2006). Demographic costs linked to the production of neckteeths might further explain the density peak observed in the *Bodrio del pastore*

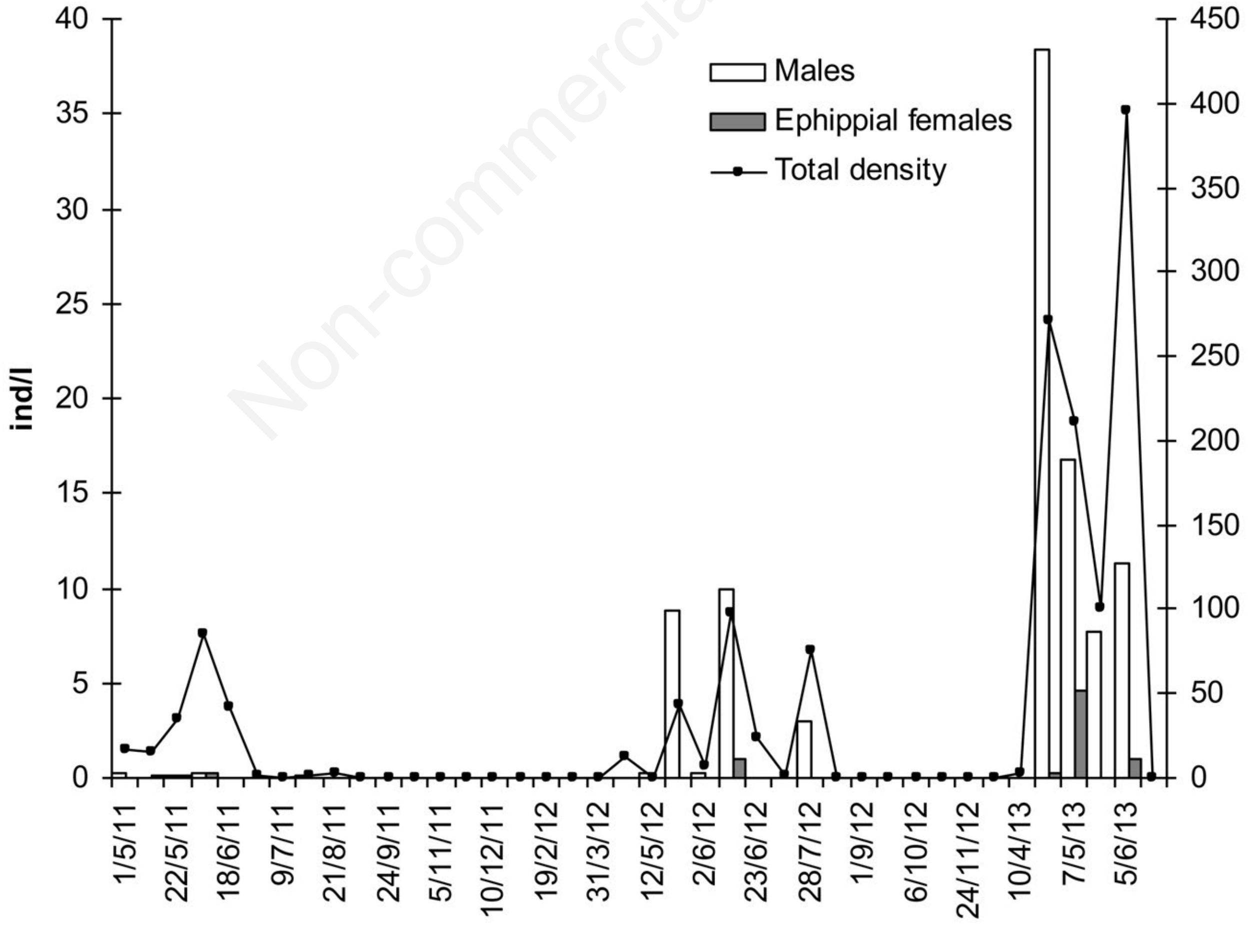

Fig. 3. Seasonal variation of total density, males, and ephippial females density in *D. pulex* population.

III population in spring 2013, when most individuals were neckteeth-less. Long tail spine may be a low cost and good start-up defence when predation pressure may change rapidly (Havel, 1985; Tollrian, 1993). The size range we observed in total length of adult females was larger than that described in *D. pulex* from Southern Italy (1310 - 2120 µm) (Leoni and Garibaldi, 2009).

As expected in populations from temperate zones, *D. pulex* from the *Bodrio del pastore III* reproduce by cyclical parthenogenesis. In most Cladocera, sexual phase and male production begin after several generations of parthenogenetic reproduction with the production of subitaneously developing eggs. Switch to sexual reproduction and resting egg production is generally cued by a number of factors that are indicative of environmental deterioration (*i.e.*, crowding and short-day photoperiod) and by maternal environmental conditions (Stross, 1987; Hobaek and Larsson, 1990; Innes and Dunbrack, 1993; Innes, 1997; LaMontagne and McCauley, 2001; Koch *et al.*, 2009). In the bodrio, a very early investment in sexual reproduction, independent of population density, was observed. A similar feature was described in *D. pulex* from two temporary ponds in Ontario and suggested a lower influence of the environmental factors on sex determination compared to populations inhabiting more permanent habitats (Innes, 1997). Since sexual reproduction is required to produce resting eggs, the ability to lay them quickly and/or continually is adaptive in an small water body whose hydroperiod is unpredictable and uncertain. During our study period, the *Bodrio del pastore III* permanently hold water but we cannot rule out that periodically it might dry out. Resting eggs are the primary dispersal stage and allow both colonisation of new sites and the persistence from year to year. Ephippial egg bank is expected to function as a reservoir of genetic diversity that may buffer populations against changes in the genotypic composition of the active population that may result from predator selection or disturbances (Spitze, 1991; Hairston, 1996). Annual recruitment of *D. pulex* from recombinant ephippial eggs guarantees the maintenance of genetic diversity and clonal variability. Lineages that produce males and ephippial females early in the season may escape from selection pressure by predators a meaningful percentage of genetic variation linked to sexual reproduction, so that evolution proceed toward earlier and increased sex allocation (Ruvinsky *et al.*, 1986; Larsson, 1991; Innes and Dunbrack, 1993). A large genetic component and local adaptation (genetic control and genotype-environment interaction) might account for the feature of cyclical parthenogenesis of *D. pulex* from *Bodrio del pastore III*.

CONCLUSIONS

Our results suggest that further investigation are advisable in order to verify whether the current population is the last remnant of a once much larger metapopulation eventually reduced by anthropogenic disturbances (*e.g.*, fish introductions, water contamination with toxic chemicals) or if it represents the invasion of North American strains that have recently colonised large parts of New Zealand, Africa and Spain, and that was already recorded in Northern Italy and Sardinia (Mergeay *et al.*, 2006;

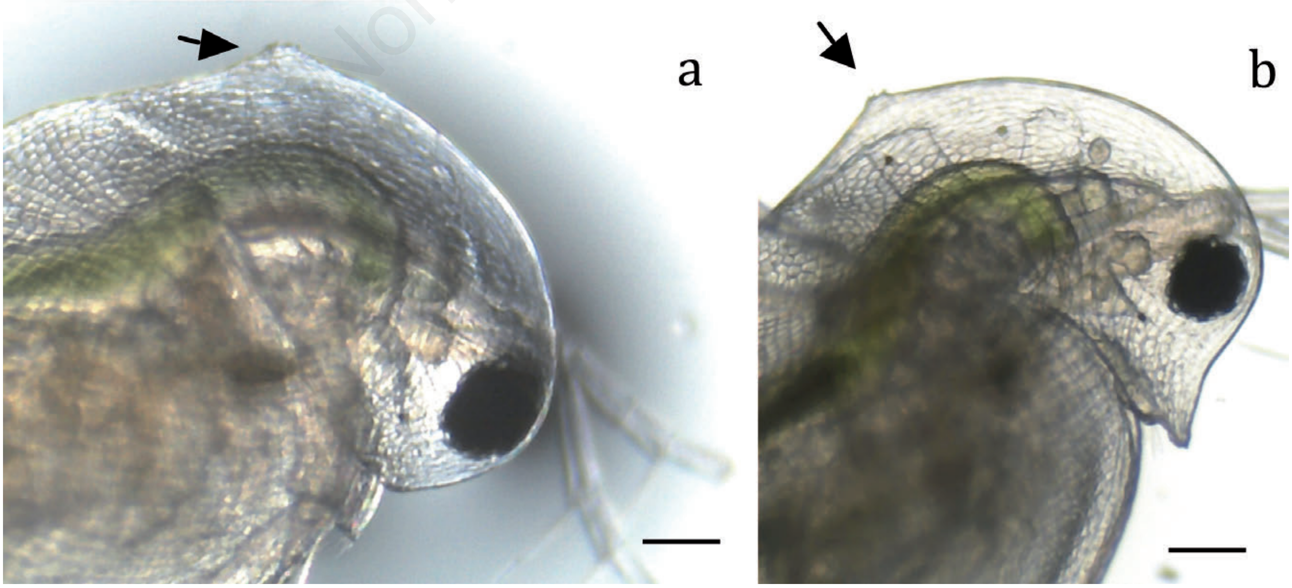

Fig. 4. Juveniles with neckteeth: a) male b) female. Arrows indicate the neckteeth. Scale bar: 60 µm.

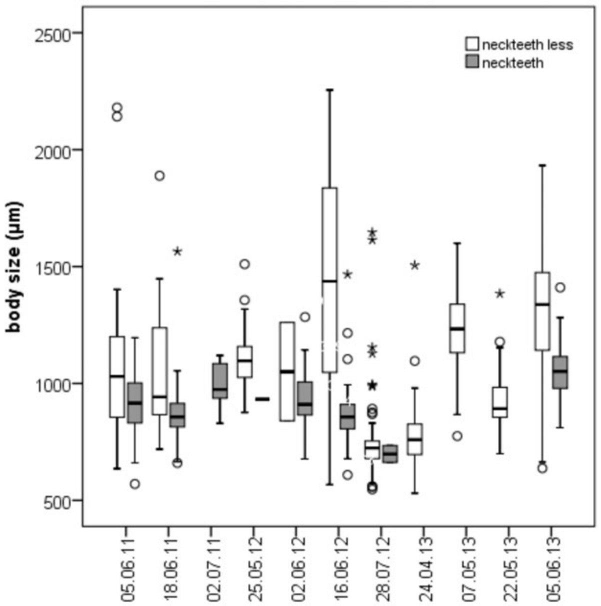

Fig. 5. Seasonal variation of body size in neckteethed and neckteeth-less females. Circles and asterisks indicate outlier data that are more than or less than 1.5-3 times (circle), or more than 3 times (asterisk) of upper or lower quartile.

Vergilino *et al*., 2011; Duggan *et al*., 2012; Crease *et al*., 2012; Markova *et al*., 2013).

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