

On the presence of *Daphnia galeata* in Lake Orta (N. Italy)

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ABSTRACT

Lake Orta is a very interesting environment because of its history of heavy chronic pollution and biota re-colonization. It was the first deep lake in Italy in which allozyme techniques were used to investigate clonal diversity. In 1986, a newly established *Daphnia obtusa* population, able to colonize stressed environments, resulted composed by just one multilocus genotype, detected through an analysis of 27 different allozymes, fifteen of which active and stable for up to 12 generations. This result was interpreted as indicative of a colonization by a single clone, able to establish after a strong selection. This species was allochthonous for the lake, and in 1996 was replaced by a *Daphnia longispina* of the same morphotype as that originally described from its pelagic environment, the latter apparently remaining the only *Daphnia* species also in the lake's littoral until 2001, i.e. until a regular sampling to monitor the lake was accomplished. During an accidental sampling in 2004, for detecting the occurrence and prevalence of parasites in *Daphnia* parental species and hybrids in several lakes located North and South of the Alps, *Daphnia galeata* was first found in Lake Orta's pelagic environment, as revealed by morphological and allozyme analyses. Lake Orta zooplankton was then re-sampled in June 2006, to detect species as well as clonal composition of the *Daphnia* population, using the same techniques of morphotype and allozyme analyses. We expected to find low clone diversity and the occurrence of hybrids, whose success is enhanced in disturbed environments. Although preliminary, our results confirm that clone diversity Lake Orta's *Daphnia* population is low, thus suggesting a recent colonization by *D. galeata* and providing evidence for a founder effect. In addition, the detection of the *M* variant at the *AO* locus, we interpret as a rare allele of *D. galeata*/*D. longispina* hybrids, perfectly fits the hypothesis of an enhancement of hybrid success in disturbed environments such as the once heavily polluted Lake Orta.

Key words: pollution, recolonization, *D. longispina*, *D. galeata* allozymes, clones, founder effect

1. INTRODUCTION

1.1. Lake Orta an elective site for studies on pollution and re-colonization

Lake Orta is an unique site for its history of pollution, recovery and re-colonization. Zooplankton, particularly *Daphnia*, has been the object of long-term investigations, which allowed for tracing the appearance, success and decline of different species. Because of its peculiar history and the need to investigate the re-colonization at high definition level (i.e. molecular, Bachiorri *et al.* 1989; Bachiorri *et al.* 1991), Lake Orta has been the first site in Italy in which a classic morphological analysis was combined with that of allozyme markers. These studies provided information on the genetic structure of the *Daphnia* population responsible for the biota re-colonization during a gradual recovery from pollution. As expected, the allochthonous *Daphnia obtusa* population detected in the lake in 1986 was genetically homogeneous, probably as the result of a successful hatching of resting eggs produced by one resistant clone, able to survive under strong selective conditions, such as those imposed by a situation of heavy pollution (Bachiorri *et al.* 1991; Bonacina *et al.* 1994).

Lake Orta case study has been a unique example of a founder effect in such a large lake and provided an

important occasion for discussing patterns and mechanisms of recovery and re-colonization of the pelagic environment. For this reason, the occasional finding of *Daphnia galeata* stimulated the present preliminary study, aimed at detecting, in addition to the presence of the species, the genetic structure of its population, as well as the occurrence of hybrids, the latter expected because of the high level of disturbance of this environment.

1.2. The study site and its history of pollution, recovery and biota re-colonization

Located in Northern Italy, Lake Orta is the seventh largest Italian lake by volume. Before pollution, the zooplankton population (Pavesi 1879; Monti 1930) was composed by seven species of cladocerans, five of copepods and several rotifers. Already poorly buffered (total alkalinity ranging between 0.3-0.4 meq l⁻¹; Monti 1929) because of the geology of the catchment basin, it was acidified after heavy industrial pollution starting from 1926 (Bonacina 2001). The pollution stopped in 1980, four years after the approval by the Italian Parliament of a law on the regulation of the discharge of industrial wastes into freshwater environments. The lake reaction was fast (Bonacina *et al.* 1986; Mosello *et al.* 1986; Bonacina *et al.* 1988a, 1988b; Calderoni *et al.* 1990a), further accelerated after a liming intervention in 1989-1990 (Calderoni *et al.* 1990b, 1991, 1992).

Zooplankton promptly reacted to the initial signals of recovery: after the rotifers, the allochthonous *Daphnia obtusa*, a species able to live in stressed environments (Fryer 1985), colonized the lake. A combined analyses of morphological and genetic diversity (Bachiorri *et al.* 1991) suggested that the population was established by a single clone, able to exploit the peculiar environmental conditions, which imposed a strong selection (Bonacina *et al.* 1994).

Strong interannual and seasonal population density fluctuations were recorded until 1996, when *D. obtusa* disappeared from the pelagic community, being replaced by a *Daphnia longispina* of the same morphotype as that found before the lake pollution (mainly identified by the absence of a dorsal head crest: Margaritora 1983; Flößner 2000; Benzie 2005).

The latter was recorded until 2001 (Signorini 2006), i.e. until when a regular monitoring of the lake zooplankton was carried on, also allowing for detecting an increasing complexity of the community along with the reduction in dissolved ammonia concentration.

In 2004, we accidentally collected live zooplankton samples, as Lake Orta was one of the 28 lakes in Italy in which a project on *Daphnia* parasites was carried out in cooperation with EAWAG, Dübendorf (Wolinska *et al.* 2007). The aim of the study was to identify and study parasite infection in parental and hybrids of the *D. longispina* group in lakes of different size and trophic history, located North and South of the Alps. The occurrence and dominance of hybrids, together with changes in lake trophy were analyzed (Keller *et al.* 2006). The results of this study, based on allozymes, indicated the presence of *Daphnia galeata* in the lake. A monitoring of Lake Orta zooplankton started again in June 2006. As *D. galeata* was still present in the pelagic zone of the lake, we also collected a live sample for allozyme analyses to investigate species, as well as clone diversity. Being Lake Orta's *Daphnia* populations cyclical parthenogens, we expected hybridization between the two most recently established species to have occurred, thus further complicating the interpretation of classic morphological analyses. In the present paper we report the results of this preliminary study.

1.3. Classic diagnostic traits and the allozyme analysis

Daphnia of the *longispina* group, to which both *D. galeata* (Sars) and *D. longispina* (O. F. Müller) belong, are the most common species inhabiting large European permanent water bodies (lakes and reservoirs). Their ubiquitous presence is attributed to their ability to cope with fish predation better than *Daphnia* of the *pulex* group (Hrbáček 1987), which tend to be confined to the littoral of deep lakes or to those with low fish predation pressure. The species of the *D. longispina* group are characterized by a high phenotypic plasticity, their highly variable morphology being strongly influenced by lake environmental conditions.

An important morphological trait for the differential diagnosis of *D. longispina* with respect to the other species of the group is the presence of a head smoothly round and with outline falling down just behind the eye, not pointed or spined, or extended into a helmet (Margaritora 1983; Flößner 2000; Benzie 2005). In addition to this relatively simple and well established criterion, other morphological traits, based on quantitative morphological features (i.e. aesthetes, ocellus, eye position and postabdominal processes) have been more recently established, which show a large variability (Petrušek *et al.* 2005), thus making the diagnosis very difficult at present. The problem of reliable taxonomical identification is further complicated by the fact that interspecific hybridisation seems to be a common phenomenon.

The occurrence of hybrids was reported from many lakes and ponds (e.g. Wolf & Mort 1986; Hebert *et al.* 1989; Schwenk & Spaak 1995; Spaak 1996) and interspecific hybrids were generated in laboratory crossing experiments (Schwenk *et al.* 2001). Despite potential introgression, parental species remain genetically distinct, since interspecific hybrids show in most cases a lower hatching and survival rate than parental species, i.e. hybrid breakdown (Schwenk *et al.* 2001). Some transient morphological forms from field samples often result from a combination of great morphological plasticity and hybridization, and therefore, molecular techniques, among which the allozyme analysis, represent an useful tool for taxonomy of field populations.

2. METHODS

Two zooplankton samples were collected in June 2006 at the station corresponding to the maximum depth of the lake (143 m) with a Clarke-Bumpus plankton sampler equipped with a 76 µm nylon net exploring a layer from surface to 50 m. One sample was immediately fixed in 90% denaturated ethanol and then transferred into a 10% formaldehyde. The other one was used to randomly select 50 live *Daphnia*, which were kept frozen in liquid nitrogen until allozyme analyses were performed. We used electrophoresis on acetate cellulose gels (Hebert & Beaton 1989), using tris-Glycine buffer at pH = 8.5. Single individuals were carefully homogenized before being loaded on the gel. Electrophoresis was carried out at room temperature at 200 volts for 18 minutes. Gel staining with the agar overlay was done at temperature of 55 °C in the thermo-incubator. Gels were scored after sufficient staining using *Daphnia* of known genotypes as standards.

We used as standards laboratory clones originated for *D. galeata* from Rimov Reservoir (Czechia) and for *D. galeata* × *longispina* from Lake Maarsseveen (The Netherlands). Four allozyme loci (sAAT: amino aspartate transferase, EC 2.6.1.1; AO: aldehyde oxidase, EC 1.2.3.1; GPI: glucose-6-phosphate isomerase, EC 5.3.1.9; PGM: phosphoglucomutase, EC 5.4.2.2) were scored. Loci were either fixed within species (differen-

Tab. 1. Allozyme phenotypes of 50 analysed *Daphnia* from Lake Orta in the sample of June 2006.

| Locus phenotype | | | | Bilocus genotype code | No of individuals |
|-----------------|----|-----|-----|-----------------------|-------------------|
| sAAT | AO | GPI | PGM | | |
| FF | MM | MM | FM | BLG – A | 49 |
| FF | MM | MM | FF | BLG – B | 1 |

tiation among taxa; sAAT and AO) or polymorphic (differentiation of multi-locus genotypes; GPI and PGM).

Randomly chosen *Daphnia* were also photographed to document *Daphnia* morphotypes. This enabled us to compare *Daphnia* from the June 2006 sample with those previously collected in May 2004.

3. RESULTS

The use of the 4 allozyme markers (sAAT, AO, GPI and PGM loci) allows a definition of a clonal diversity in 50 specimens of *Daphnia*. sAAT (synonym to GOT) was first reported by Wolf & Mort (1986) as a diagnostic marker to discriminate between *D. galeata* and *D. longispina*. *D. galeata* is fixed for F (fast) alleles, whereas *D. longispina* is fixed for S (slow) alleles at this locus, therefore making the distinction between the two very easy. Lately, Giebler (1997) reported AO as a second diagnostic marker, as *D. galeata* appeared to be fixed for F (fast) alleles at this locus, while *D. longispina* was fixed for the S (slow) alleles. These two diagnostic markers typically enable a discrimination of the species, and of the backcrosses and second generation's hybrids as well. To solve the clonal attribution the other two additional alleles (GPI and PGM), which are polymorphic, are typically used.

The results of our analyses indicate an extreme homogeneity of the material (Tab. 1). The species diagnostic locus sAAT indicated unambiguously the presence of *D. galeata* genes (all specimens were homozygous for F allele). The result of AO, the second species diagnostic locus, was rather intriguing, as all specimens were homozygous for M; this allele can be interpreted as a marker for *D. cucullata*, or as a rare allelic variant of *D. longispina* and of *D. galeata*.

The homogenous character of the analysed sample was found also on both presumed polymorphic loci GPI and PGM. GPI locus was fixed for the M allele. PGM locus showed heterozygous FM combination for all specimens except for one, which was FF homozygous. The resulting clonal structure of the sample indicates that 49/50 specimens belong to the clonal group FFMMMMFM (sAAT + AO + GPI + PGM) and only one specimen has the variant FFMMMMFF.

The homogenous character of the sample was also confirmed by the microscopic analysis of *Daphnia* phenotypes, which resulted very uniform (June sample) (Fig. 1 d, e, f) and consistent with a pure *D. galeata* morphotype. As most individuals in the sample were subadult, we had to include 10 pre-adults in addition to the 40 ovigerous females present. However, no adult *Daphnia* registered and photographed before analysis

resembled the *D. longispina* phenotype recorded in the past. In May 2004, both *D. galeata* and *D. longispina* were morphologically identified, in a proportion of 18% and 82% respectively. Therefore, the prevalence of *D. galeata* became clearer in 2006, as no *D. longispina* could be identified even on morphological basis. The two sibling species reached very similar levels of abundance in the two different periods (2530 and 2812 ind m⁻³ in May 2004 and in June 2006, respectively).

4. DISCUSSION AND CONCLUSIONS

The presence of *D. galeata* in the nearby lakes might explain its appearance in Lake Orta, both by natural vectors or through transport associated with human activities.

The opportunity for an immigrant genotype to colonize the habitat depends on whether resources have been monopolized by resident genotypes, the intrinsic growth rate of the population, the carrying capacity and the number of colonizing propagules (De Meester *et al.* 2002). While the success of allochthonous species may be reduced by competition with locally adapted resident genotypes (De Meester 1996), the colonization is enhanced by the unstable conditions characterizing Lake Orta.

The results of allozyme analysis on Lake Orta *Daphnia* unambiguously confirm the presence of *D. galeata* genes, according to the presence of F allele at sAAT locus. However, they also allow a discussion on the relevance and possible interpretation of AO-based species diagnosis. In particular, the presence of allele M can be interpreted in different ways. It has been suggested (Giebler 1997) that this allele is indicative of the presence of *Daphnia cucullata* genes. According to what we know on the ecology of this species, this is not a reliable interpretation for Lake Orta because *D. cucullata* is considered a "lowland" species, typical of well buffered water bodies (Fryer 1985). In addition, according to the results by Keller *et al.* (2006), *D. cucullata* appears distributed mainly north of the Alps, therefore allowing a distinction of two biogeographic regions, a northern one in which *D. hyalina* and *D. cucullata* prevail, and a southern one, with higher temperatures and lower latitudes, in which *D. galeata* dominates.

According to the current taxonomical keys, the morphological diagnosis for this species is unequivocal, especially in live adults, because of the lack of an ocellus, and the emergence of the antennular aesthetes from a rounded rostrum tip.

To interpret the results of our analysis of Lake Orta *Daphnia* we favour the alternative interpretation of the

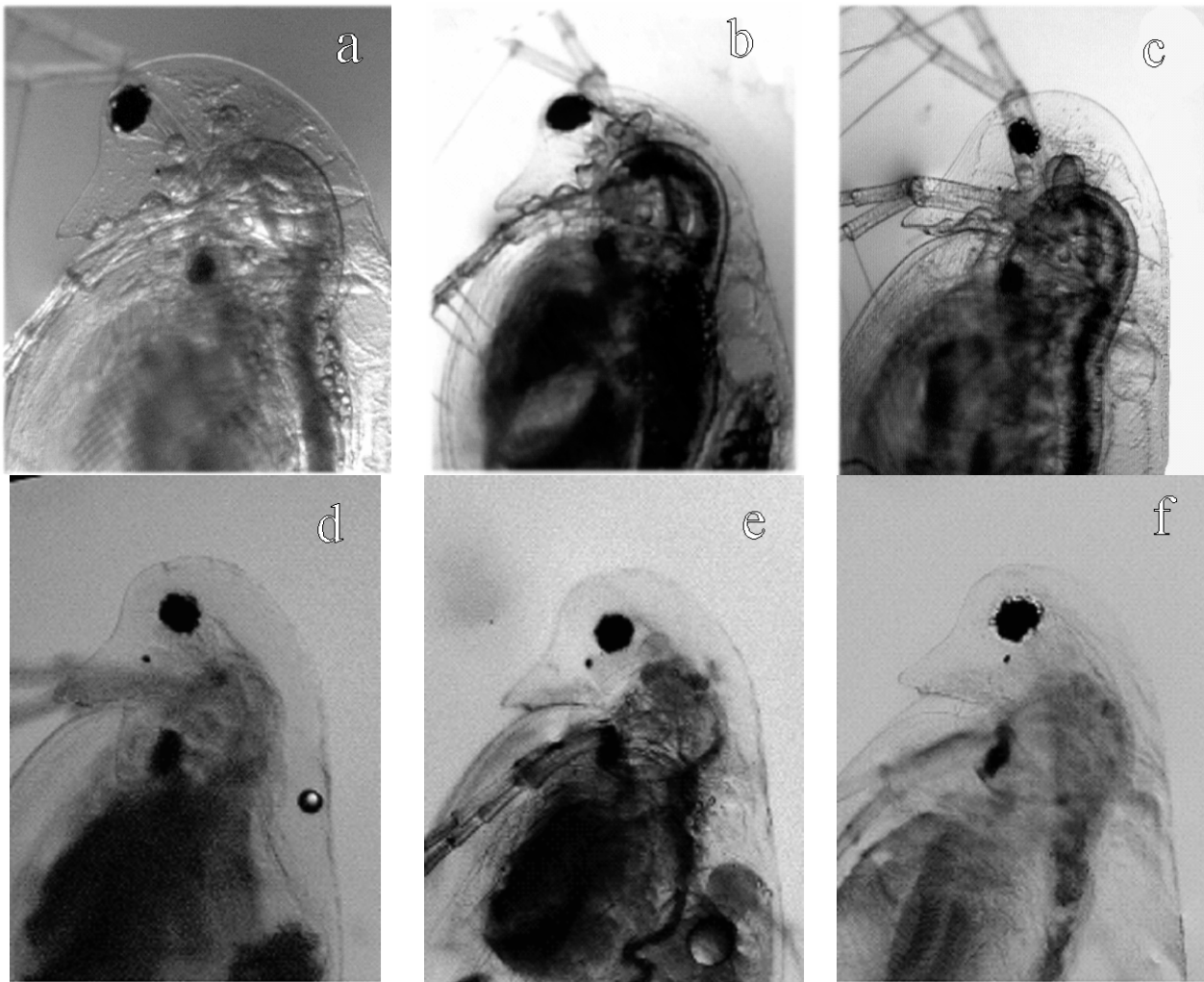


Fig. 1. Comparison of *Daphnia* morphotypes from May 2004 (a - c) and June 2006 (d - f) samples. Note the ventral position of the eye cavity in photographs a and b, which is one of the discriminating characters for *D. longispina*.

allele at AO locus as a rare *D. galeata* variant, or as indicative of second generation's *D. galeata* × *longispina* hybrids. The two hypotheses are equally supported by our analysis. Our findings are in relatively good agreement with those of Keller *et al.* (2006) who in 2004 identified as *D. galeata* allozyme phenotypes about 50% of Lake Orta's *Daphnia*. The persistence of hybrid generations is also coherent with the *Daphnia* population dynamics in Lake Orta (Signorini 2006): presence of males is not a rare phenomenon, being detected in spring as well as in autumn, thus providing a good opportunity for the production of hybrids.

In nearby Lago Maggiore, despite the non-regular appearance of *Daphnia* males, the presence of *D. hyalina* × *galeata* hybrids, in addition to the two parental species, was first detected after the lake re-oligotrophication (Manca *et al.* 1996).

In addition, the fact that GPI locus was fixed for M allele, an uncommon situation for this relatively highly polymorphic locus, may be interpreted as resulting from

a founder effect. Furthermore, the high heterozygosity level at PGM locus, with a FM combination for 49 specimens out of 50 analyzed *Daphnia* may be interpreted as a strong clonal selection of heterozygotes. The alternative explanation of an absence of sexual reproduction does not seem coherent with the occurrence of males we regularly observed in Lake Orta *Daphnia* population.

Therefore, the Lake Orta case study would confirm what was observed in other lakes of the region. As emphasized by Keller *et al.* (2006) habitat disturbances, such as changes in trophic, enhance hybridization. Chronic pollution and recovery may be regarded as a habitat disturbance, as well as a stress (Cattaneo *et al.* 1998). While in general hybrids seem to perform poorly compared with their parental species (Schwenk *et al.* 2001), under specific environmental conditions they may have equal or higher fitness.

Notably, the second generation hybrids cannot be distinguished from their parents on a morphological basis.

The absence of *D. longispina*-like morphotypes in the June samples may suggest a replacement of *D. longispina* by *D. galeata* in summer. However, we do not have a whole year collection of samples, as the zooplankton monitoring started again only in June 2006.

The extreme homogeneity of *Daphnia* for the loci GPI and PGM can be interpreted as the combination of a founder effect and a strong clonal selection.

This result is in agreement with population genetic theory, which predicts that newly established populations will exhibit founder effects, including loss of genetic variation and/or alteration of genetic structure from passing through a bottleneck, if the founding population is small (Boileau *et al.* 1992; DeMelo & Hebert 1994). The same homogeneity was found for *D. obtusa* by Bachiorri *et al.* (1991): all the 1341 *D. obtusa* specimens analyzed between 1987 and 1989 resulted monomorphic for 15 loci. This result supported the idea that the lake was colonized by a single clone (if not by "a single egg"!), particularly resistant to the specific environment, therefore providing an example of a founder effect with a strong clonal selection in a highly disturbed environment.

A strong clonal (or species) selection may be also deduced from the very few data on morphology we have obtained from microscopic analysis (cf Fig. 1).

Finally, the presence of *D. galeata* in Lake Orta provides an additional evidence for the improvement of environmental conditions which started from the eighties with an increasing complexity of the zooplankton community (Fig. 2).

The present manuscript is a preliminary note about the presence of *D. galeata* in Lake Orta. Further studies are needed for better understanding its appearance, its future development and its implication for the structure and functioning of the lake food web.

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