

Reproduction experiment on *Austropotamobius pallipes* complex under controlled conditions: Can hybrids be hatched?

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Received January 28, 2011

Revised March 30, 2011

Accepted April 19, 2011

ABSTRACT

Key-words:
reproduction,
hybrids,
Austropotamobius
pallipes
complex,
controlled
conditions

The *Austropotamobius pallipes* complex is formed by the two species *A. pallipes* and *A. italicus*, recently defined only on genetic basis, without obvious morphological features for their differentiation. Dealing with this problem, we have conducted a breeding experiment between these two presumed species under controlled conditions, in order to investigate the possible existence of reproductive barriers. Using 80 breeders (56 females and 24 males) collected from populations where their species identity was genetically well established, we performed four series of trials: $F_{ita} \times M_{pal}$ and $F_{pal} \times M_{ita}$ interspecific mating; $F_{ita} \times M_{ita}$ and $F_{pal} \times M_{pal}$ intraspecific mating as a control. We have monitored only the main phases of the reproduction in order to avoid excessive interference for the crayfish. In November 2009 the 91.7% of females having intraspecific mating, as well as the 100% of females having interspecific mating, produced eggs. In June 2010 the eggs from the 36.4% of females having intraspecific mating, and those from the 42.3% of females having interspecific mating did hatch. We emphasize the hatching of hybrids and their vitality.

RÉSUMÉ

Expérience de reproduction du complexe *Austropotamobius pallipes* en conditions contrôlées : les hybrides peuvent-ils éclore ?

Mots-clés :
reproduction,
hybrides,
complexe
Austropotamobius
pallipes,
conditions
contrôlées

Le complexe *Austropotamobius pallipes* est formé de deux espèces, *A. pallipes* et *A. italicus*, récemment définies sur des bases génétiques, sans caractères morphologiques évidents pour les différencier. Pour aborder cette question, nous avons conduit une expérience de reproduction entre ces deux espèces présumées en conditions contrôlées, de façon à rechercher l'existence possible de barrières de reproduction. Avec 80 reproducteurs (56 femelles et 24 mâles) collectés dans des populations où l'identité spécifique était bien établie, nous avons conduit quatre séries d'essais : $F_{ita} \times M_{pal}$ et $F_{pal} \times M_{ita}$ croisement interspécifique ; $F_{ita} \times M_{ita}$ et $F_{pal} \times M_{pal}$ croisement intraspécifique comme contrôle. Nous avons suivi seulement les phases principales de la reproduction pour éviter un dérangement excessif des écrevisses. En novembre 2009, 91,7 % des femelles après croisement intraspécifique et 100 % des femelles après croisement interspécifique ont produit des œufs. En juin 2010, les œufs de 36,4 % des femelles à croisement intraspécifique et 42,3 % des femelles à croisement interspécifique ont éclos. On insiste sur l'éclosion des hybrides et leur vitalité.

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INTRODUCTION

The endangered white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) is already identified as a species complex (Souty-Grosset et al., 2006). Early studies based on morphological characters (Bott, 1950, 1972; Karaman, 1962; Brodsky, 1983) disagreed on taxonomic level and names; then in recent years many molecular studies focused on this controversial status (Santucci et al., 1997; Lörtscher et al., 1998; Gouin et al., 2001; Grandjean et al., 2000, 2002).

The current situation, based on 16S mtDNA analyses, shows that *A. pallipes* and *Austropotamobius italicus* (Faxon, 1914) as two species occur in Italy and the latter one differentiated in four subspecies: *A. i. carinthiacus* in Central and NW Italy; *A. i. carsicus* in NE Italy; *A. i. italicus* on the Tuscan-Emilian Apennines (Central Italy); *A. i. meridionalis* in S Italy (Fratini et al., 2005). Genetic studies focused on the Po River drainage revealed the presence of both *A. pallipes* and *A. italicus* (Zaccara et al., 2004) and an overlapping area in the province of Alessandria (NW Italy), where the two species were found to be sympatric in at least two of the brooks examined (Ghia et al., 2006).

However, studies based on morphological and/or morphometric data are few and haven't revealed a distinctive character that would separate two species (Ghia et al., 2006; Bertocchi et al., 2008).

Furthermore, even if most molecular studies show high nucleotide divergence between *A. pallipes* and *A. italicus* (see Grandjean et al., 2000; Fratini et al., 2005) and then a genetically clear separation between the two species, possible hybridisations cannot be assessed with the matrilineal molecular marker (mtDNA) used in these phylogeographic studies.

Before making a clear decision on the specific status of a group, morphological, biological and phylogenetic data must be considered together (Avice and Ball, 1990).

According to the most recent Mayr's definition of biological species as "groups of interbreeding natural populations that are reproductively (genetically) isolated from other such groups" (Mayr, 2004), on one hand we can deem *A. pallipes* and *A. italicus* as two different species due to their genetic distance, but on the other hand we do not recognize if the two species are able to interbreed. Preliminary research on breeding behaviour of *A. pallipes* complex suggests a probable reproductive isolation (Russo et al., 2004; Ghia et al., 2006).

Therefore the purpose of this work is to investigate the reproductive interactions of the two Italian taxa of *Austropotamobius pallipes* complex under controlled conditions: (1) to investigate possible differences in the reproduction phases; (2) to test whether and at which level of reproductive isolation the two species appear to be; (3) to further determine the relationship between *A. italicus* and *A. pallipes*.

MATERIALS AND METHODS

> BREEDING CENTRE CONDITIONS

Our study was conducted from 12 October 2009 to 15 September 2010, in an experimental indoor breeding centre located in the Regional Park "Alto Garda Bresciano" in Lombardy (N Italy), that was already proved to have good breeding conditions during progress of a previous Life Project (Fea et al., 2007a). Groups of crayfish were placed in eight opaque tanks (each 70 L × 50 W × 50 H cm), fed continuously with running water from an adjacent spring. Optimum water conditions were always verified along the experimental stage; water velocity was varying but providing good oxygenation, median O₂ 99.8% (range 96.6–107.2), median O₂ 10.6 ppm (range 8.7–11.9). Water hardness was about 205 mg·L⁻¹ in calcium carbonate, pH in the range 7.8–8.4 (median 8.22) and median conductivity was 325 μS·cm⁻¹ (range 319–336). The water temperature was recorded using Tinytag[®] temperature logger (Gemini Data Loggers, UK Ltd) at 2 h intervals and varied from 2.3 to 22.7 °C (mean 11.98 °C, median 11.2 °C).

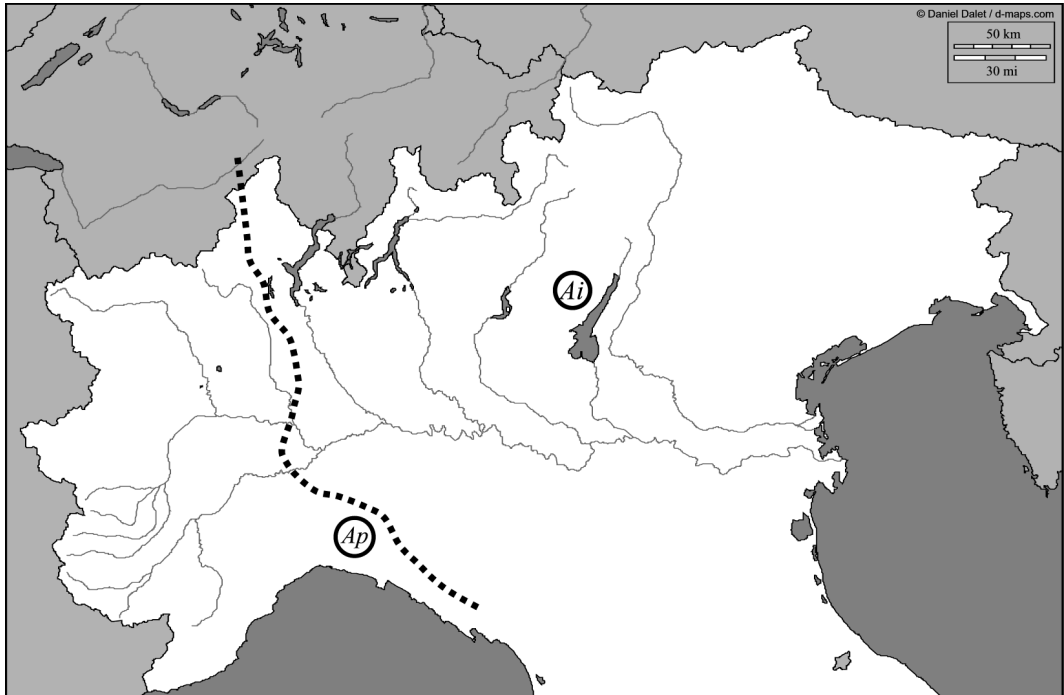


Figure 1

Sampling sites of the two populations belonging to *A. pallipes* complex; the black dotted line represents the border between *pallipes* (*Ap*) and *italicus* (*Ai*) in Northern Italy. (Downloaded from http://www.d-maps.com/carte.php?lib=italia_del_nord_cartina&num_car=5882&lang=it and modified according to Zaccara et al., 2005.)

Figure 1

Sites d'échantillonnage de deux populations appartenant au complexe *A. pallipes*; la ligne en pointillés représente la frontière entre *pallipes* (*Ap*) et *italicus* (*Ai*) au nord de l'Italie. (Téléchargé depuis http://www.d-maps.com/carte.php?lib=italia_del_nord_cartina&num_car=5882&lang=it et modifié selon Zaccara et al., 2005.)

Each tank was equipped with a multi-hole brick (40 L × 25 W × 18 H cm) to act as a shelter for the crayfish (Ghia et al., 2009) and with bundles of twigs to make it more natural.

The crayfish were maintained under natural light conditions; they were fed twice a week until December and after March, once a week from December to March as all crayfish need less food due to the drop in temperature; crayfish were fed jellied chironomids, grated carrots and feedstuff for benthic fish and crustaceans.

> EXPERIMENTAL DESIGN

A total number of 80 breeding crayfish was collected by hand during night time at the end of September 2009 from two populations (Figure 1), chosen from previous studies, where their species identity was genetically well established (Zaccara et al., 2004; Ghia et al., 2006; Fea et al., 2007b): 40 specimens of *A. pallipes* from the Lemme basin (Eastern Piedmont) and 40 specimens of *A. italicus* from the Toscolano basin (Eastern Lombardy). Twenty-eight females and 12 males were collected from each site. Female receptivity was assessed by the presence of the glair glands (Taugbøl and Skurdal, 1989), visible as a whitened tissue ventrolaterally on the abdominal somites (Reynolds, 2002), and male maturity by their total length (Nardi et al., 2004; Galeotti et al., 2006).

In early October 2009 the total length of the breeding crayfish was measured using a digital calliper (accuracy ± 0.1 mm); they were marked on the telson using the branding method developed by Abrahamsson (1965) in order to distinguish *pallipes* from *italicus* without causing

the crayfish too much interference. Additionally within the species they were individually numbered on the cephalothorax by means of a waterproof pen. In October 2009 females and males together were placed in the eight tanks according to the following four trials: $F_{ita} \times M_{ita}$ and $F_{pal} \times M_{pal}$ as intraspecific mating; $F_{ita} \times M_{pal}$ and $F_{pal} \times M_{ita}$ as interspecific mating. Each trial was carried out on 14 females and 6 males distributed in two tanks (7 females and 3 males per tank).

During the mating phase, at each monitoring check, the remaining unfertilised females (not having spermatophores and/or eggs) were re-mated with other males according to the trial. After spawning, the egg-bearing females were held in common tanks all the time and the males were taken away from the mating tanks to avoid disturbance and aggressive contacts with females and therefore loss of pleopodal eggs (Reynolds, 2002). In June 2010, after eggs hatched, females were kept with juveniles for few weeks more until they were independent, due to prolonged brood care in freshwater crayfish, carrying also the early juvenile stages for weeks or even months under the mother's abdomen (Whitmore and Huryn, 1999; Vogt and Tolley, 2004).

We have monitored only the main phases of the reproduction in order to avoid excessive interference for the crayfish, because heavy losses of eggs had been recorded during the first period of embryogenesis due to handling and poor egg attachment, as described by Celada et al. (1988), Woodlock and Reynolds (1988a), Carral et al. (1994, 2000) and Celada et al. (2001).

In September 2010, at the end of the experiment, all breeding crayfish were returned to their original streams, while juveniles were maintained in the breeding centre for future studies.

> STATISTICAL ANALYSES

In order to investigate the fecundity, egg-bearing females were divided into four groups according to number of eggs visually assessed during each monitoring: none (not fertilized or loss of eggs), low (< 15 eggs), medium (15–40 eggs), high (> 40 eggs). These categories were chosen according to the very low fecundity estimated from pleopodal eggs counts in *A. pallipes* complex (Brewis and Bowler, 1985; Reynolds, 2002). We performed Chi-square test to determine whether there were any differences between the number of females having interspecific mating (as observed counts) and females having intraspecific mating (as expected counts).

Non-parametric statistical tests were carried out when the scores appeared to be drawn from a not normally distributed population (Sokal and Rohlf, 1995); statistical differences were detected with a level of significance $p < 0.05$ and they were performed with SPSS software (ver. 16.0).

RESULTS

Total lengths of females were not significantly different between the two tanks (t) of the same trial (*pallipes* intra [t1 vs. t5]: Kolmogorov–Smirnov $Z = 0.802$, $p = 0.541$; *italicus* intra [t2 vs. t6]: $Z = 0.535$, $p = 0.938$; *pallipes* inter [t3 vs. t7]: $Z = 0.535$, $p = 0.938$; *italicus* inter [t4 vs. t8]: $Z = 0.802$, $p = 0.541$), so the data were combined and four groups of females were considered in the following analyses. Furthermore females' total length was not different in intra- and interspecific mating, neither for *pallipes* (intra vs. inter: Kolmogorov–Smirnov $Z = 1.134$, $p = 0.153$) nor for *italicus* (intra vs. inter: Kolmogorov–Smirnov $Z = 0.567$, $p = 0.905$).

Almost seven months (since mid October until early June) passed between the mean time of spawning and of hatching, calculated as 1391 CTU (Celsius temperature units = degrees \times days; 193 days at mean water temperature of $7.1 \text{ }^\circ\text{C} \pm 3.79$). In general for both intra- and interspecific mating, the graph (Figure 2) shows mating and spawning period, egg-bearing and post-hatching period in relation to the temperature.

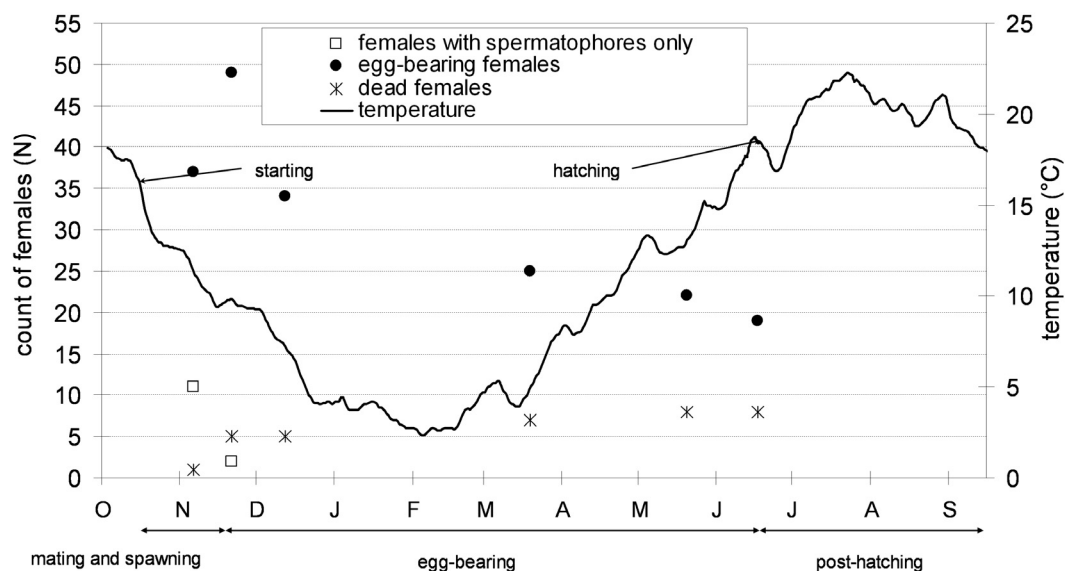


Figure 2

Main phases of *A. pallipes* complex reproduction and post-hatching period in relation to the mean daily water temperature during the experiment. Temperature line is showed on the secondary y-axis.

Figure 2

Phases principales de la reproduction du complexe *A. pallipes* et période post-éclosion en relation avec la température moyenne journalière de l'eau pendant l'expérience. La température est portée sur l'ordonnée secondaire.

By mid November 2009 most females (96.1%) were fertilized and bore eggs (Table I), except two *pallipes* females involved in intraspecific mating, which remained unfertilized until mid December 2009 despite the fact that the other *pallipes* males were placed with them. All trials showed a decline in numbers of females from the beginning to the end of the experiment (mortality: *A.p.* intra 21%; *A.i.* intra 21%; *A.i.* inter 14%), except *pallipes* females having interspecific mating (0%). Interspecific mating trials revealed some significant differences (see Table I) in females frequencies comparing to intraspecific mating trials, but frequencies of the latter ones were almost always lower.

There were no statistic differences in the *pallipes* females' fecundity (Figure 3) between females that mated intra- and interspecific (March: $\chi^2 = 5.776$, $p = 0.056$; May: $\chi^2 = 4.511$, $p = 0.105$), except in December ($\chi^2 = 9.011$, $p = 0.029$) when there were significantly more intermating females with medium and low egg counts. Anyway we highlight on one hand the occurrence of egg-bearing females having interspecific mating; on the other hand the trend of egg loss by all *pallipes* females.

As regards *italicus* females' fecundity (Figure 4), we did not find any statistic differences between females that mated intra- and interspecific (December: $\chi^2 = 3.575$, $p = 0.311$; March: $\chi^2 = 3.131$, $p = 0.372$; May: $\chi^2 = 4.241$, $p = 0.237$); on the contrary, we stress that the egg-bearing females having interspecific mating are always the most numerous ones.

Moreover there was no significant variation of females' total length among fecundity classes in different months (Kruskal–Wallis test in *italicus* always $p \gg 0.302$; in *pallipes* always $p \gg 0.063$).

Testing all data sets between *pallipes* and *italicus*, we found significant differences in fecundity (December: $\chi^2 = 14.868$, $p = 0.002$; March: $\chi^2 = 23.709$, $p = 0.000$; May: $\chi^2 = 24.223$, $p = 0.000$). Only in December there was a significant difference in total length between *pallipes* and *italicus* egg-bearing females (Kolmogorov–Smirnov test, December: $Z = 1.455$, $p = 0.029$; March: $Z = 1.311$, $p = 0.064$; May: $Z = 0.905$, $p = 0.387$).

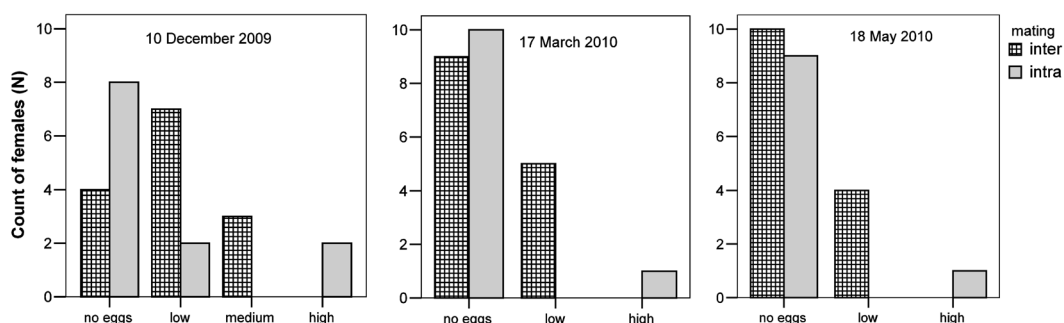


Figure 3
Distribution of *A. pallipes* females among fecundity classes, compared between intra- and interspecific mating.

Figure 3

Répartition des femelles d'*A. pallipes* selon les classes de fécondité, comparaison entre croisement intra- et interspécifique.

Table 1

Mating trials and long-term outcomes. Frequencies are presented as ratio of fertilized or egg-bearing females and alive ones. Chi-square test was performed to determine whether there were any differences between the number of females having interspecific mating (as observed counts) and females having intraspecific mating (as expected counts). "Fertilized" = having spermatophores and/or eggs; "nv" = not valuated.

Tableau 1

Essais de croisement et résultats à long terme. Les fréquences sont présentées comme la proportion de femelles fécondées ou porteuses d'œufs et œufs éclos. Le test Chi-carré est fait pour déterminer les éventuelles différences entre le nombre de femelles à croisement interspécifique (comme observé) et le nombre de femelles à croisement intraspécifique (comme escompté). « Fertilized » = femelle ayant des spermatophores et/ou des œufs; « nv » = non estimé.

	♀ × ♂ starting	♀ fertilized	♀ fertilized	♀ fertilized	♀ egg-bearing	♀ egg-bearing	♀ egg-hatched
<i>Trial</i>	12/10/09	4/11/09	19/11/09	10/12/09	17/03/10	18/05/10	15/06/10
♀ <i>Ap</i> × ♂ <i>Ap</i>	14 × 6	9/13	10/12	4/12	1/11	1/11	1/11
♀ <i>Ap</i> × ♂ <i>Ai</i>	14 × 6	12/14	14/14	10/14	5/14	3/14	0/14
χ^2 test; <i>p</i>		0.181	nv	0.002	0.000	0.104	nv
♀ <i>Ai</i> × ♂ <i>Ai</i>	14 × 6	13/14	12/12	8/12	7/11	7/11	7/11
♀ <i>Ai</i> × ♂ <i>Ap</i>	14 × 6	14/14	13/13	12/13	12/13	11/12	11/12
χ^2 test; <i>p</i>		nv	nv	0.052	0.033	0.046	0.046

In mid June 2010 eggs did hatch, from both *italicus* females having intraspecific mating (63.6%), and from ones having interspecific mating (91.7%). Whereas eggs hatched only from *pallipes* females having intraspecific mating (9.1%).

In mid September 2010 the offspring were counted and measured (Table II); juveniles total length was not different as regards cross mating (Kolmogorov–Smirnov test, in *italicus*: $Z = 1.099$, $p = 0.179$). We stress out the vitality of *italicus* hybrids and the fact that they were the most numerous ($\chi^2 = 5.910$, $p = 0.015$).

DISCUSSION

All four mating trials took place at almost the same time in October, when water temperature rapidly decreased, but it was always recorded when water temperature was above 10 °C. Values as high as 13.5 °C recorded in Spain (Carral *et al.*, 1994) agree with our results, on the contrary temperatures below 10 °C were recorded in Ireland (Woodlock and Reynolds, 1988b). Mating and spawning occurred during a short period (24 days) and most females carried both

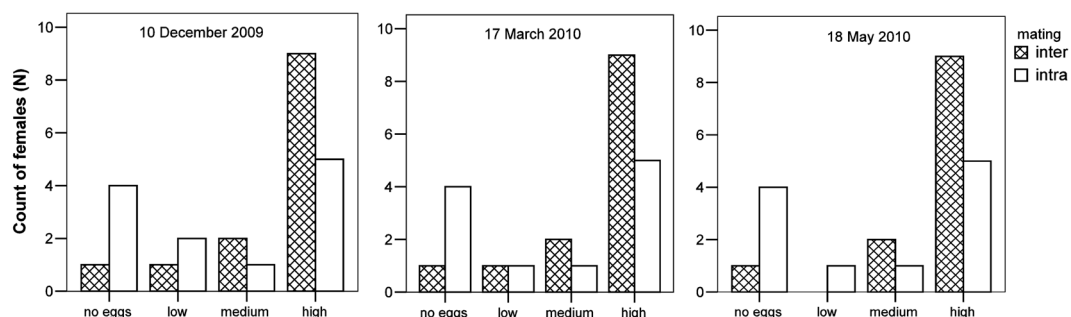


Figure 4

Distribution of *A. italicus* females among fecundity classes, compared between intra- and interspecific mating.

Figure 4

Répartition des femelles d'*A. italicus* selon les classes de fécondité, comparaison entre croisement intra- et interspécifique.

Table II

Mean total length of the *A. pallipes* complex juveniles three months old. Number of juveniles (*N*) and standard deviation (*Std. dev.*) are given as well.

Tableau II

Longueur totale moyenne des juvéniles d'*A. pallipes* du complexe, âgés de trois mois. Nombre de juvéniles (*N*) et déviation standard (*Std. dev.*) sont indiqués.

Offspring from	<i>N</i>	Mean <i>TL</i> (mm)	<i>Std. dev.</i>
♀♀ <i>Ap</i> × ♂♂ <i>Ap</i>	5	16.20	1.304
♀♀ <i>Ap</i> × ♂♂ <i>Ai</i>	0	–	–
♀♀ <i>Ai</i> × ♂♂ <i>Ai</i>	27	19.07	2.235
♀♀ <i>Ai</i> × ♂♂ <i>Ap</i>	35	18.00	1.627

spermatophores and eggs already at our first check, agreeing with data of 4–6 days between mating and spawning (Reynolds *et al.*, 1992). Also, upper temperature limits vary among populations of *Astacus astacus* in relation to local climatic conditions (Taugbøl and Skurdal, 1990); it was shown that combination of drop in temperature and daylight decrease induces the beginning of reproduction (Reynolds, 2002).

All females were fertilized with a M:F constant ratio of 1:2.3, in accordance with many authors (see Taugbøl and Skurdal, 1989; Carral *et al.*, 1994; Mackevičienė *et al.*, 1999; Sáez-Royuela *et al.*, 2005); only two females (3.6%) remained unmated, even if they were assessed receptive. This proportion was small and is comparable to 4.3% of the females, which had undeveloped ovaries (Taugbøl and Skurdal, 1989). In natural environment proportion of mature females varies according to inter-annual changes (Neveu, 2007) and it appears smaller than those observed in aquaculture studies (Taugbøl *et al.*, 1988).

Just a month after spawning, many egg-bearing females lost eggs, particularly *pallipes* that had intraspecific mating (up to 60%); since females were assessed with spermatophores, eggs were regarded fertilized and were lost due to different reasons, such as disturbance and progressive thinning of the egg pedicel (Reynolds, 2002). Egg loss was significantly different between females having intraspecific and interspecific mating, but variation of 27–28% in favour of the interspecific mating let us suppose that the choice of male species did not at all contribute to the reproductive success. Other factors, such as the feeding regime during ovarian development (Sáez-Royuela *et al.*, 2006) which occurred in original streams, should be considered. Number of pleopodal eggs and survival rate are higher in females mated in group than in those individually inseminated (Sáez-Royuela *et al.*, 2005); this is possibly due to sperm contribution from more males (Galeotti *et al.*, 2007).

However we highlight the occurrence of egg-bearing females having interspecific mating, both in *pallipes* and in *italicus* until May, a period corresponding to the embryonic phase of the eyes (Celada *et al.*, 1991). In order to further investigate genetically the hybrids, we took egg samples from each egg-bearing females in May, including those bearing only few eggs, and therefore we decreased probability of hatching success.

Despite last egg loss, we achieved the main purpose of the study: in June offsprings did hatch, either from females having intraspecific or interspecific (only *italicus* females) mating. Such a goal was never reached so far in studies on *Austropotamobius* genus, while Kossakowski (1966) and Degiorgi *et al.* (1994) reported achievement of hybrids between *Astacus leptodactylus* and *A. astacus*. In another experiment (Holdich *et al.*, 1995), where cross mating success between *A. pallipes*, *A. leptodactylus* and *Pacifastacus leniusculus* was studied, all eggs were lost a few weeks after spawning. Exceptions were *A. leptodactylus* females mated with *A. pallipes* or *P. leniusculus* which retained a few undeveloped eggs for 20 weeks (Holdich *et al.*, 1995).

> IMPLICATIONS IN BIOLOGICAL CONSERVATION

In our study mating success of *A. pallipes* complex, either in intraspecific or interspecific mating, showed no reproductive barrier and the isolation mentioned in previous researches (Russo *et al.*, 2004; Ghia *et al.*, 2006) does not work at pre-zygotic level, because at least one female having one out of two interspecific trials carried eggs during 26 weeks. As regards post-zygotic isolation, *italicus* hybrid zygotes were formed and successfully developed in three months post-hatching period. In order to know whether they are sterile, we are growing them up in the breeding centre for the required period to become sexually mature (three years at least!).

Reproductive interference is a plausible species replacement mechanism, especially in indiscriminately mating species like crayfish (Butler and Stein, 1985). In North America, for example, *Orconectes rusticus* x *Orconectes propinquus* hybrids are fertile and backcross largely with *O. rusticus*; therefore, as pointed out by Perry *et al.* (2001), genetic mechanisms have contributed to the displacement of *O. propinquus* by *O. rusticus*. Usually the invader mates with females of the resident species in order to remove the female from that year's reproduction (Reynolds, 2002) and cohabitation of more species in a stream is a transitional phase, after which only one species is found (Smith, 1981; Hogger, 1988).

Continuing with the starting hypothesis that *A. pallipes* complex is formed by the two species, we could consider *A. pallipes* as the invader species due to its successful mating with *italicus* females. Recent research showed that Italian and French *pallipes* populations share same haplotypes and it was suggested that human translocation events occurred throughout the Alpine barrier and that *A. pallipes* might have replaced the native *A. italicus* on the east side of the Maritime Alps (Stefani *et al.*, 2011). *A. pallipes* individuals used in our experiment were collected from a *pallipes* population placed next to boundaries of its eastern distribution area, so we could suppose that some hybrids were already living within the population.

Reproductive interference constitutes a serious threat for the survival of the geographically isolated populations, with the risk of extinction caused by hybridisation of endangered populations.

Further studies are required to improve the present intricate matter, taking into account a larger sample of crayfish from the core of their distribution areas and using diagnostic nuclear markers on populations living in the overlapping of the two distribution areas. For example, morphological and genetic data strongly support the identification of the fragile shelled mussels as a mixture of *Mytilus trossulus* and *M. trossulus* x *Mytilus edulis* hybrids in UK seawaters (Beaumont *et al.*, 2008).

CONCLUSIONS

The assumption that *A. pallipes* and *A. italicus* exist as two distinct species is weakened. So, on one hand, we have to pay the utmost attention in reintroduction and restocking actions of the conservation programmes, in order to avoid endangering the genetic integrity of existing populations, especially in sympatric areas; on the other hand we can assume that the two species are “separately evolving metapopulation lineages” (de Queiroz, 2007) at speciation level, during which current species concepts come into conflict.

ACKNOWLEDGEMENTS

We are grateful to Bruna Comini and Paolo Nastasio (ERSAF – Regione Lombardia) for allowing the experimental study in the breeding centre in the Parco Alto Garda Bresciano; Giuseppe De Franceschi for his help in taking care and feeding of the crayfish; Carlo Violani for assistance in the translation of a first draft of the paper.

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