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Divergent demographic patterns and conservation of endemic species in extreme environments: the case study of the spring snail Melanopsis etrusca (Gastropoda: Melanopsidae)

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1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
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Divergent demographic patterns and conservation of endemic species in extreme environments: the case study of the spring snail *Melanopsis etrusca* (Gastropoda: Melanopsidae).

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Abstract

The conservation of endangered species poses challenges driven by contrasting targets and needs. Populations' isolation can indeed be a value or a limitation due to promoted evolutionary change and risk of bottlenecks and/or other genetic detrimental processes.

In the present study we illustrate the case of *Melanopsis etrusca* (Brot, 1862), an endemic snail commonly associated with some thermal springs from Central Italy. These habitats represent a climatic refuge in response of dramatic paleo-climatic events.

Populations are currently isolated within headstream stretches by the gradual changes in physico-chemical conditions downstream. Furthermore, strong differences in spring thermal regimes have a role in determining population dynamics and life history, as showed by differential patterns of individual growth and mortality.

Snails experiencing temperate conditions with relatively high temperature fluctuations (13-29 °C) showed seasonal patterns of growth with good level of age-cohorts structuration. Conversely, fast and continuous growth, with shorter life cycles, is typical of snails constantly inhabiting warmer streams (31-34°C). Interestingly, population structures similar to those observed in cooler conditions were recognized in a hot channel (36-38°C), where snails spend long period outside the water, thus exploring an additional thermal niche.

Furthermore, a partial shift of snails' diet reflects in terms of epilithic communities, which change towards cyanobacteria-dominated environment in warmer streams.

Hot-adapted populations of *M. etrusca* appear to have larger potential for recolonization after habitat perturbation or population collapse events, although they may be at risk of bottlenecks. On the other hand, the isolation in climatic refuges seems to have promoted behavioral strategies to cope with extreme thermal environments.

In a general perspective, thermal streams appear valuable model systems to investigate patterns of populations' adaptation and divergence, as those triggered by climate change.

1. Introduction

Freshwater mollusks are among the most endangered metazoan groups, representing almost 42% of the total recorded animal extinctions in the last 500 years (Lydeard et al. 2004). Such a trend appears determined by several factors among which scarce mobility of these animals, frequent water-bodies isolation (Strayer 2006; Olden et al. 2010) and general limited resilience of freshwater habitats affected by anthropogenic pressure (e.g. Lysne et al. 2008).

Such threatens appear magnified for endemic species limited to springs habitats, often characterized by particular physicochemical conditions, which may impose the development of specific physiological/behavioral adaptations to survive extreme conditions. These habitats are considered biodiversity hotspots (Di Sabatino et al. 2003) due to the very peculiar invertebrate and fish community herein hosted (e.g. Vinyard 1996; Hershler et al. 2007).

Freshwater prosobranchs belonging to the genus *Melanopsis* are widespread all around the Mediterranean basin where this group had a long evolutionary history. *Melanopsis* fossils from brackish/marine deposits are known since the lower Cretaceous (e.g. Stewart et al. 1991). However, the largest radiation of the genus occurred at the end of the Miocene during the Messinian Mediterranean salinity crisis (about 5 Ma), when brackish basins were abundantly present in several regions (e.g. Esu 1980; Esu & Girotti 1989; Geary 1990; Guerra-Marchán et al. 2010).

Within this genus, *M. etrusca* is a small (15-20 mm in shell length), poorly known endemic species whose relict populations dwell few thermo-mineral springs in Southern Tuscany (Central Italy; Glaubrecht 1993). Climatic shifts through glaciation events are commonly recognized as one of the main extinction factors of the closest ancestors, once abundant and diversified in this geographic area, until the early Pleistocene (e.g. Esu et al. 1993; Di

Bella et al. 2002). However, in the last century, relict populations of this species underwent further extinctions due to anthropogenic pressure (Cianfanelli et al. 1989). Exploitation of thermal springs for spas as well as therapeutic, wellness and fitness activities, draining and contamination of aquifers related to agricultural and/or mining purposes can be regarded as the primary causes for the populations loss (Cianfanelli et al. 1989). Owing to its critical status, *M. etrusca* has recently been classified as 'Endangered' by IUCN red list of threatened species (Cianfanelli 2010).

The almost complete lack of knowledge about general aspects of the biology of this species renders difficult to propose any effective actions for its protection (e.g. Watson & Ormerod 2004). Furthermore, the investigation of life history traits in populations adapted to diverging environmental conditions provides basic knowledge for both conservation and understanding of organisms' potentials for acclimatization and adaptation in evolutionary terms. This appears particularly worthy considering the challenge posed to freshwater habitats and species conservation by the rapid human-induced climatic changes (Heino et al. 2009; Woodward et al. 2010).

The present study was aimed to clarifying possible physico-chemical constraints of *M. etrusca* distribution, the importance of such parameters to its life-history as well as aspects of its trophic biology. Such issues were addressed by comparing *M. etrusca* grazing, patterns of individual growth and population structure in different physico-chemical conditions, both along the same stream and between streams.

2. Material and Methods

2.1 Study area

M. etrusca occurs in six localities (Venturina, Aronna, Bruna, Podere Bagnolo, Poggetti Vecchi and Roselle; Fig.1) of southern Tuscany characterized by thermo-mineral waters (Bencini et al 1977; Celati et al 1991), two of which (Poggetti Vecchi and Podere Bagnolo) are severely endangered by anthropogenic brook desiccation (Fig. 1).

In the present study the following sites were considered: Venturina, Aronna, Bruna and Roselle (Fig. 1). The thermal regime of the springs from which water courses originate is quite different, since it varies from constantly high temperatures (>30°C), as the stream at Venturina and the thermal channel at Roselle, to cooler (<30°C) sites such as those of Bruna and Aronna.

The streams show narrow riverbeds (1.5-4 m) with shallow waters (5-40 cm). The current regimes are from fast/moderate to very slow on hard (cobbles, gravels, rocky beds) and soft (muddy) substrates, respectively.

M. etrusca is mainly distributed in the very first hundreds meters of the streams, while in downstream areas this species is only occasionally observed, likely due to current dragging of isolate specimens.

2.2 Physical and chemical parameters of the waters

Sampling stations were selected in order to identify the main physico-chemical parameters accounting for the typical restricted distribution of snails into headstream positions. For each stream, two sampling stations were selected: 1) upstream (hereafter called 'upstream'), where dense assemblages of *M. etrusca* occurred and 2) downstream (hereafter called 'extinction'), where local extinction of the population was observed.

Four water samples were collected at each of the described locations, between August 2009 and May 2010. Water samples were collected and filtered (0.45 µm) and transferred into polyethylene bottles for the subsequent chemical analyses in laboratory. Temperature (°C) and pH were determined in the field with an Hg thermometer and a portable Eutech PC650 waterproof instrumentation, respectively.

The main anions and cations were analyzed by ion chromatography (761 Compact IC-Metrohm and 861 Advanced Compact IC-Metrohm respectively). Analytical errors were generally <3% for the main components. TDS (Total Dissolved Solids) was referred to the sum (in mg/L) of the cationic and anionic species.

2.3 Population structure, individual growth and mortality rates

Population structures from all the four sites were assessed on two different moments of the year, July 2009 and February 2010, in stream stretches with hard substrate and similar conditions in terms of depth, current and distance from the spring. Snails density and size were measured, as follows: a specific sampler was built by using a 20×20 cm plastic bottle opened at both ends. The "cylinder" had a wall mounted with a thin conic aquarium mesh (0.2 mm), whereas the opposite one was punched. The sampler was placed on the stream bottom and the enclosed surface scraped with a brush. As a consequence, snails and other material in suspension were naturally pushed into the net by the current flowing through the punched wall. A soft rubber-made rib fixed onto the lower edge allowed a complete adherence of the sampler to the riverbed.

Eight quadrates, assigned to two transects, were sampled in each stream. This appeared to be a reasonable amount of replicates considering both the very high abundance of snails and the relative uniformity of the riverbed conditions. An additional sampling was carried out at Roselle (hereafter Roselle-*Amph*), in a headstream stretch were an atypical, massive amphibious behavior was observed, with inactive specimens forming a dense belt along the stream bank, few centimeters above the water level.

The collected material was carefully cleaned. Snails were then placed on a plastic tray with their shell opening turned up and a picture was taken for successive specimens counting and size estimates. The snails were released at the end of this procedure.

Picture analyses were carried using the free software Image-J (available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). Such an indirect method of estimation was necessary due to the local very high density of snails.

Growth rates were measured in semi-natural conditions, by considering different thermal environments. The Bruna stream and Venturina channel were thus selected as experimental sites hosting cool and warm adapted populations of *M. etrusca*, respectively.

Four 26×10×16 cm plastic boxes (hereafter "mesocosms") were placed in each stream in the same localities of population structure sampling. Large windows of thin zinced mesh were present on each side of the mesocosm, except the bottom, in order to allow a constant water flow inside (Fig. 1). Mesocosms were set up with cobble collected from the riverbed. A total of 30 snails were introduced in each mesocosm. Shell length ranged between 3.1 and 16.8 mm for Bruna and between 3.1 and 10.8 mm for Venturina, representing the natural differences in maximum size reached by the two populations. Each animal was individually tagged (n=120) by coloring the top of the shell with several chromatic codes by using permanent markers.

The experiment started at the beginning of September 2009 and ended in July 2010.

Increment in shell height and shell width was monthly measured until November, again after two months, in January 2010, and subsequently every 3 months (accuracy 0.01 mm). Every time, the number of dead specimens inside the mesocosms was recorded and mortality rates were computed.

2.4 Food items on hard substrates/crop contents

An explorative study was carried out through a semi-quantitative method in order to investigate the main microphytobenthic groups naturally present on streams cobbles and to perform a comparison with the food items ingested by *M. etrusca*

An overall surface of 130 cm^2 was sampled from each site in unshaded homogeneous riffle stretches (depth 5-10 cm) densely populated by *M. etrusca*, following the general indications given in the European standard EN 13946:2003 for epilithic diatoms. Samples were obtained by randomly scraping single 13 cm^2 units from cobbles and stones surfaces by means of a steel blade. Collected samples were preserved into 4% formalin solution and carried to laboratory for taxonomic identification (Bouchard & Anderson 2001). For each site, six aliquots of 50 µL were observed into 3 mL sedimentation chambers, using an inverted microscope (Zeiss IM) with 400x magnification. Sampling was carried out in August 2009 and February 2010.

M. etrusca gut contents were obtained by dissecting (Bilgin 1973) 20 snails collected during August 2009, in the same area of cobbles collection of two sites characterized by different thermal conditions: Bruna and Venturina. For each site, five 50 µL subsamples of the pooled extract were observed similarly to what described for phytobenthic analysis.

2.5 Data analysis and statistics

Principal Component Analysis (PCA) was used to visualize patterns of variability among samples and to determine the contribution of each single physicochemical parameter to the overall observed variability. Permutational analysis of variance (PERMANOVA; Anderson 2001) was used to test the null hypothesis of no differences in normalized physicochemical water parameters, among the four sites (factor "Site": four levels, random and orthogonal) and position along streams (factor "Position": two levels, fixed and orthogonal). Similarly, the hypothesis of no difference between shell length increments (normalized on the initial size) among sites with different thermal regimes was tested, by considering the first month of experiments. The following factors were considered: "Site" (two levels: Bruna and Venturina; fixed and orthogonal); "Position" (two levels: upstream and extinction; fixed and orthogonal); "Mesocosm" (four levels; random and nested in "Position").

Permutations of residuals under a reduced model (more than 9900) were based on similarity matrices computed on Euclidean distances of normalized data and Type III sum of squares.

The SIMPER procedure (Clarke 1993) was used to find out the percentage contribution of the overall variability observed among samples. Univariate statistical analysis was applied to the most relevant variables and carried out by using ANOVA. The same models as in the multivariate analysis were considered. Cochran's C-test was performed to test the normality of distributions prior of performing ANOVAs and square root transformations were used when opportune. SNK-test was used to test for differences among levels of factors found to be significant by ANOVA.

Average size and density of snails from summer samplings were analyzed by ANOVA, taking into account two factors: "Site" (four levels; fixed) and "Transect" (two levels; random and nested in "Site"). The stretch including amphibious specimens in Roselle was not included in the analysis due to the different patterns of animal occurrence that is mainly outside the 'riverbed'. For both PERMANOVA and ANOVA, square-root transformation of data was carried out when appropriate.

Chi-square test of independence was used to compare the absolute abundance of the main microphytobenthic groups between two sites characterized by different thermal regimes (Bruna and Venturina). A similar analysis was carried out to compare microalgal occurrence in snail crops and natural substrates. Pooling of data was carried out when appropriate (minimum expected frequencies less than 5).

Multivariate analyses of variance were performed using PRIMER v. 6.1 (Clarke & Gorley 2006) and PERMANOVA+ for PRIMER routines (Anderson et al. 2008). ANOVAs were performed using the software GMAV-5 for Windows (Underwood et al. 2002).

Age cohorts identification was carried out following the method of Battacharya (1967), using FiSAT (FAO-ICLARM Stock Assessment Tools, www.fao.org/fishery/) free software (Gayanilo et al. 1996).

3. Results

3.1 Physico-chemical water parameters

Streams strongly differed in terms of thermal regime. During the study period water temperature of Bruna stream underwent seasonal variations of around 12 °C (13.7-25.7 °C) at upstream position and together with Aronna (19-22.7 °C), represented the two cooler streams. Conversely, Venturina and Roselle were characterized by temperatures constantly above 30 °C. The highest temperature were reached at Roselle-*Amph* (≥38°C,

mean value = 37 °C). The observed ΔT between upstream and extinction positions during the sampling period varied among different sites and according to the period of the year (Tab. 1).

The values of the main physico-chemical water parameters are listed in Table 1. The sampled waters at both the upstream and extinction positions were characterized by relatively high values of TDS from 1,381 mg/L (Aronna Ext) up to 2,678 mg/L (Roselle Ups). The main cations were Ca^{2+} and Mg^{2+} whose concentrations were up to 592 mg/L (Roselle upstream) and 125 mg/L (Roselle upstream), respectively. Among the anionic species SO_4^{2-} and HCO_3^- showed the highest contents with concentrations up to 1,664 mg/L (Roselle upstream) and 280 mg/L (Bruna upstream), respectively. According to the 'Venice System' (1959) such a kind of water can be classified as oligohaline (e.g. Por 1972; Hartog 1974). The bulk of variability among samples was explained by a geographic factor, determining a clear segregation of samples in the multivariate space (Fig. 2). A general decreasing trend of water temperature (Tab. 1)(riportare i valori) and main dissolved anions and cations species was observed moving away from upstream positions (Tab.1), whereas pH levels showed a rising pattern towards exctinction positions.

A significant interaction was found by PERMANOVA between distance and position (Tab. 2): in particular, pairwise comparisons performed for the different sites found significant differences between upstream and extinction position in each stream, except for Bruna (PERMANOVA, post-hoc tests).

SIMPER found pH, HCO_3^-, NO_3^-, K^+ and temperature explaining almost 76% of the differences observed between upstream and extinction positions averaged over different sites. However, the univariate analysis showed pH being the only parameter significantly changing at the extinction positions across streams (ANOVA, SNK test; Tab.3). On the other hand, NO_3^- was found to be significantly higher only at the extinction positions of Venturina and Roselle (ANOVA, SNK test; Tab. 3).

3.2 Population density and size class distribution
Populations of *M. etrusca* were characterized by very dense assemblages, with peaks of over 10,000 snails per square meter, as estimated from some quadrates in Roselle (Fig. 3A). Population from the warmest sites achieved the highest average densities, although significant differences were found only between Roselle and the two cooler streams, Bruna and Aronna (ANOVA, SNK test; Tab. 4).

The analysis of age cohorts performed with Bhattacharya method (Fig. 4A; Tab. 5), showed significant differences among the various populations. During summer, the population from Bruna appeared well-structured, presenting at least four age cohorts. In winter they were reduced to three, with a pick of small specimens. Population from Aronna showed to be less structured with two age cohorts both in summer and winter (Fig. 4C; Tab. 5). An almost non-structured population was recorded at Venturina, with a first well-defined cohort and a second one scarcely represented in summer, while in winter only one cohort was recognized (Fig. 4D; Tab. 5). A similar pattern was observed at Roselle, although a second cohort was also found in winter (Fig. 4E; Tab. 5). For both these sites, in winter, the first cohort appeared shifted towards smaller average sizes (Tab. 5). Interestingly the population structure for the amphibious population at Roselle appeared to be quite well-structured, with three age cohorts during both seasons and with a well-represented occurrence of large specimens (Tab. 5).

3.3 Population structure, individual growth and mortality rates
After the first month of measurements, significant differences in growth rates were found across positions of different sites and between upstream and extinction positions at Venturina, whereas these did not significantly differ at Bruna (Fig. 5A; PERMANOVA, post-hoc tests; Tab. 6).

M. etrusca individual growth from Bruna showed, in both positions, a clear seasonal pattern (Fig. 5A). Actually, the starting average shell length increment measured at the end of summer dropped to almost zero in the period October-January. Between January and April 2010, upstream individual growth recovered to a level similar to the one measured at the end of summer, although slightly lower, probably due to the ageing of specimens jointed to the progressive reduction of the experimental replicates. It must be noted that the value reported in Figure 5A is averaged over three months, thus hiding possible differences between the start and the end of the interval. Conversely, the reprise of growth at the extinction station was delayed, being still absent in April 2010.

At Venturina, the first measured length increment for the upstream population (September-October 2009) was more than twice the growth measured in the other stream (Fig. 5A). At this site, the average values of shell increment quickly decreased, which is probably to be again related to the quick ageing of the population inside the mesocosms.

Specimens moved at the extinction position showed a null growth during the first month (PERMANOVA, post-hoc tests), followed by a slight reprise in November 2009 and a new,

definitive drop, mainly determined by the strong reduction of the experimental replicates. A strong mortality indeed affected the specimens moved at the extinction of Venturina, which, on average, after only two months reached almost 80% (Fig. 5B). In the upstream position, on the contrary, such a value was attained after seven months. In Bruna stream, a different pattern was observed, with an average mortality lower than 30% after 10 months in the extinction position and lower than 5% in the upstream position (Fig. 5B).

3.4 Food items on hard substrates/crop contents

The microscopic analysis of the microalgal communities revealed pennate diatoms and cyanobacteria, these two groups being the most common constituents of hard riverbeds. The comparison of periphyton composition between Bruna and Venturina showed opposite patterns. In particular, in both sampling datess, diatoms represented the dominant group in the Bruna stream with a low occurrence of cyanobacteria. On the contrary, Cyanobacteria appeared the dominant group at Venturina (Chi-square test of independence, August 2009: χ^2= 486.98, df=1, p<0.001; February 2010: χ^2=337.87, df=1, p<0.001; Fig. 6A). Similar patterns, although less pronounced, were shown at the Aronna stream and Roselle.

Despite the consistent quantities of fine detritus, the gut content of *M. etrusca* showed a large amount of macrophyte fragments and a smaller portion of microphytobenthic items (cyanobacteria, diatoms and rare green algae). In particular, Bruna and Venturina had no statistical differences between the relative abundance of the microalgal items in the snail's gut and in the natural substrates (Chi-square test of independence, Bruna: χ^2=0.62, df=1, p=0.43; Venturina: χ^2=2.17, df=1, p=0.14). On the contrary, the gut content significantly differed between the two sites, with specimens from Bruna largely ingesting more macrophyte tissues (87.5%) and diatoms (9.7%), whereas in Venturina, blue-green algae (48.5%) and macrophytes fragments (47.7%; Fig. 6B; Chi-square test of independence, χ^2=152.43, df=2, p<0.001).

4. Discussion

Our results showed that temperature have a role in shaping life cycles of the isolated populations of *M. etrusca* in Southern Tuscany. Snails needed to cope with heterogeneous thermal microhabitats met within different spring systems. However, temperature alone is

not able to fully explain the observed differences in population dynamics among streams as observed at Venturina extinction position.

The effect of the progressive change in water physico-chemical parameters appeared less evident due to both the large variability found among sites and the different responses obtained in the growth experiments. The most relevant parameters distinguishing between upstream and extinction positions were pH and, partially, NO_3^-. The latter was significantly higher at Venturina and Roselle, notably the two locations more affected by human activity (intensive agriculture, thermal spa and urbanization).

Snails moved to the 'extinction' position in Bruna exhibited only a delay in the seasonal reprise of growth compared to animals at upstream location, however maintaining similar rates. At this site, the multivariate analysis did not show significant differences in parameters between upstream and extinction, except for an increase in pH. The slight difference in temperature between the two stations could likely be the cause of the observed patterns, especially considering the thermoregulatory effect of the springs located on the bottom of the Accesa Lake, whose Bruna is an effluent. On the contrary, concerning the extinction at Venturina, despite still relatively elevated temperature (20.1-31.3 °C), growth was partially suppressed and high mortality was observed. Here, similarly to the extinctions at Roselle and Aronna, the progressive decrease in water temperature and dissolved CO_2, which tends to equilibrate with that of the air, determined a significant increase of pH and a consequent precipitation of travertine. This phenomenon caused the mesocosms, and the cobbles inside, to be fully coated with a carbonatic film, possibly limiting food availability. Similarly, specimens occurring only periodically and at very low density in the extinction position of Aronna (possibly dragged off by stream current) were unable to properly retreat inside the shell, due the thick concretion covering the operculum. Generally speaking, if the role of hosmoregulatory limitations should be investigated, given the brackish/oligohaline environments of *M. etrusca* ancestors (Esu & Girotti 1989; Stewart et al. 1991; Cipollari et al. 1999; Di Bella et al. 2002), other environmental factors could concur in limiting the current distribution of the species. It is known that pattern of freshwater distribution along a stream can be predicted by the progressive change of current speed and substrate (Dillon 2000). In particular, similarly to other spring snails (Martinez & Thome 2006), *M. etrusca* seems to prefer moderate currents and hard substratum (Aquiloni et al. unpubl. data).

At upstream positions, the differences in thermal regimes between streams were reflected by differential rates of snails' growth and mortality. The relatively rapid shell accretion rates (0.03 mm/d, in the first two months) observed for the population dwelling in Venturina (30-35.1 °C), nearly matched those reported for *Pyrgulopsis bruneauensis*, a hydrobiid spring snail experiencing similar thermal conditions in Idaho (0.03 mm/d at 34 °C for 89 days, for juveniles specimens; Mladenka & Minshall 2001). Growth performance of *M. etrusca* appeared to be enhanced and continuous in hotter environments (Huey & Kingsolver 1989). Nevertheless, in such conditions, average smaller specimens size and an almost complete lack of age structure was observed.

M. etrusca at Bruna showed the capability of tolerating seasonal drop in temperature (13.7 °C in the present study, but lower in other years: SIRA, 2011) through reduction/suspension of growth. Our data showed a reduction in shell accretion in October 2009 – January 2010, with a reprise, at an undefined time, between January and April 2010. The average shell increment of 0.7% referred to February, March and April 2010, is indeed only an indirect estimate and the reprise of growth can reasonably be hypothesized at the achievement of a certain environmental temperature threshold. Such a temporary reduction of growth likely appears to be an effect of a general metabolic depression, similarly observed in other overwintering species of freshwater mollusks (Lurman et al. 2014). In winter, age cohorts structuration appeared less evident and this could be a bias due to the tendency, especially of larger animals, to rest below large boulders (pers. observ.): an additional clue of low temperature-induced diapause. Such an annual dynamics appears to determine longer life cycles, as demonstrated, in our mesocosm experiment, by low, discontinuous growth and little mortality. These evidences well matched the observed higher level of age structuration exhibited by the Bruna population, where seemingly long-standing specimens were found (maximum recorded shell length: 15.9 mm). Conversely, the high mortality rates of Venturina seem to account for shorter life cycles with faster specimen's turnover and potentially higher generation rates in hot streams.

Interestingly, we observed a massive amphibious behavior performed by (large) snails inhabiting a headstream area in Roselle, where the highest water temperatures occurred (34.6-38.1 °C). Here, differently than in other locations of the same channel, as well as at Venturina, a more structured population was observed, despite the warmer conditions. A reduced average body temperature experienced throughout the year by these animals, determined by the exploration of a different thermal niche, could account for the observed

population structure. Evidences from population dynamics of *M. etrusca* naturally exposed to different thermal environments seem to verify the general *temperature-size rule* (e.g. Atkinson et al. 2006), which predict lower temperature causing the majority of ecthoterms to growth slower and attaining maturation at larger body size.

Eventually, temperature also appeared to affect microalgal epilithic communities and, in turn, this had a role in shaping snails' diet. *M. etrusca* pattern of periphyton grazing indeed appeared to be not a selective one. Blue green algae attained almost half of the total food item in the stomach of specimens from Venturina, reflecting the general pattern of cyanobacteria enhancement and diatoms reduction within warmer stream.

In general, as for the congeneric *M. praemorsa* (Heller & Abotbol 1997), leaf litter of terrestrial origin appeared one of the most prominent item ingested by *M. etrusca*. However, if the low trophic values of cyanobacteria are considered (Demott & Müller-Navarra 1997), sometimes even avoided by certain freshwater snails (Madsen 1992), it is possible that the observed differences in diet may have some impact on *M. etrusca* life cycle.

Food availability is known to be a key factor for the development of effective physiological response to stressful conditions (e.g. Thomsen et al. 2013), like those potentially met in the warmest streams. Future studies should deepen the actual rates of ingested items assimilation, as well as the nutritional value of detritus in the snails' crop.

In a conservation perspective, populations dwelling warmer sites seem to hold higher recovery potential from human induced local extinctions, such as those determined by water contamination, brock desiccation or riverbed dredging. Some of the studied streams indeed fall in urban areas (Venturina, Roselle) and, more in general, all of them are virtually interested by periodic interventions of dams and riverbeds management. On the other hand, the risk of bottleneck events appears a serious threaten to the conservation of *M. etrusca* (e.g. Martin et al. 2012), given the present absence of genetic flow among populations.

It is known that the need to cope with different selective pressures (such as extreme temperatures) promotes population responses, ranging from simple phenotypic buffering to truly evolutionary adaptations (e.g. Reusch 2014). This could be the case of the amphibious behavior exhibited by a portion *M. etrusca* population in Roselle (Bartolini and Giomi unpubl. data). Such a divergent pattern would indeed make this population an ESU (Evolutionary Significant Unit), thus valuable as conservation target (Crandall et al. 2000).

In such a scenario, there is a need of genetic studies, which can quantify the actual level of divergence among the Tuscan populations (and relative to other circum-Mediterranean entities; Glaubrecht 1993) and consequently establish conservation priorities. Behavioral experiments would also be helpful, in order to establish, from an evolutionary point of view, the degree of novelty of the amphibious behavior exhibited by a portion of Roselle's population. Management of isolated populations may actually pose challenges driven by contrasting targets and needs. The necessity of guaranteeing appropriate levels of genetic variability would require actions such as cross-introduction programs (Liu & Hersher 2012), whereas for preserving any evolutionary acquisitions and innovations, the priority would be that to maintain populations' isolation.

M. etrusca represents an intriguing case study for investigating freshwater ectotherms patterns of physiological and behavioral adaptations along temperature clines. In general, our study places emphasis on the need of conserving rare and localized populations of endemic/isolated ectothermic species, such as those found within spring habitats. Their study seems indeed to hold the potential of clarifying the evolutionary processes and adaptive trajectories undertaken by isolated population in response to extreme or limiting environments. Furthermore, thermal springs are recognized as valuable model systems to assess responses of lotic communities and species under climate change (Friberg et al. 2009).

References

Anderson MJ 2001. A new method for non-parametric multivariate analysis of variance. Austral. Ecol. 26: 32–46.

Anderson MJ, Gorley RN & Clarke KR 2008. PERMANOVA+ for PRIMER: Guide to software and statistical methods. PRIMER-E Ltd, Plymouth. 214 pp.

Atkinson D, Morley SA & Hughes RN 2006. From cells to colonies: at what levels of body organization does the 'temperature-size rule' apply? Evol. Dev. 8: 202–214.

Bencini A, Duchi V & Martini M 1977. Geochemistry of thermal springs of Tuscany (Italy). Chem. Geol. 19: 229-252.

Bhattacharya CG 1967. A simple method of resolution of a distribution into Gaussian components. Biometrics. 23: 115–135.

Bilgin FH 1973. Studies on the functional anatomy of *Melanopsis praemorsa* (L.) and *Zemelanopsis trifasciata* (Gray). Proc. Malacol. Soc. Lond. 40: 379-393.

Bouchard RW, Anderson JA 2001. Description and protocol for two quantitative periphyton samplers used for multihabitat stream sampling - Documentation of periphyton sampling techniques employed by the Central Plains Center for Bioassessment. Central Plains Center for Bioassessment, Kansas Biological Survey - University of Kansas.

Celati R, Grassi S, D'Amore F & Marcolini L 1991. The low temperature hydrothermal system of Campiglia, Tuscany (Italy): a geochemical approach. 20: 67-81.

Cianfanelli S 2010. *Melanopsis etrusca*. In: IUCN 2011. IUCN Red List of Threatened Species. Version 2014.3. <www.iucnredlist.org>. Accessed 26 February 2015.

Cianfanelli S, Talenti E & Calcagno M 1989. Le stazioni di *Melanopsis dufouri* Ferrusac, 1823 (Gastropoda, Prosobranchia) in Italia. Quad. Mus. St. Nat. Livorno. 10: 59-76.

Cipollari P, Cosentino D, Esu D, Girotti O, Gliozzi O & Praturlon A 1999. Thrust-top lacustrine–lagoonal basin development in accretionary wedges: late Messinian (Lago-Mare) episode in the central Apennines (Italy). Palaeogeogr. Palaeocl. 151: 149–166.

Clarke KR 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18: 117–143.

Clarke KR & Gorley RN 2006. PRIMER v6. User Manual/Tutorial. PRIMER-E Ltd, Plymouth. 190 pp.

Crandall KA, Bininda-Emonds ORP, Mace GM & Wayne RK 2000. Considering evolutionary processes in conservation biology. Trends Ecol. Evol. 15: 290-295.

Demott WR & Müller-Navarra DC 1997. The importance of highly unsaturated fatty acids in zooplankton nutrition: evidence from experiments with *Daphnia*, a cyanobacterium and lipid emulsions. Freshwater Biol. 38: 649-664.

Di Bella L, Carboni MG & Bergamin L 2002. Pliocene-pleistocene foraminiferal assemblages of the middle and lower Tiber valley: stratigraphy and paleoecology. Geol. Romana. 36: 129-145.

Di Sabatino A, Cicolani B & Gerecke R 2003. Biodiversity and distribution of water mites (Acari, Hydrachnidia) in spring habitats. Freshwater Biol. 48: 2163–2173.

Dillon Jr RT 2000. The Ecology of Freshwater Molluscs. Cambridge University Press, Cambridge. 509 pp.

EN-13946 2003. Water quality—Guidance standard for the routine sampling and pre-treatment of benthic diatoms from rivers. European Committee for Standardization, Brussels. 14 pp.

Esu D 1980. Neogene freshwater gastropods and their evolution in the western Mediterranean area. Geol. Romana. 19: 231-249.

Esu D & Girotti O 1989. Late Miocene and Early Pliocene continental and oligohaline mollscan faunas of Italy. Boll. Soc. Paleont. Ital. 28: 253-263.

Esu D, Girotti O & Kotsakis T 1993. Palaeobiogeographical observations on Villafranchian conti-nental molluscs of Italy. Scripta Geol. Spec. Issue 2: 102-119.

Friberg N, Dybkjær JB, Olafsson JS, Gislason GM, Larsen SE & Lauridsen TL 2009. Relationships between structure and function in streams contrasting in temperature Freshwater Biol. 54: 2051–2068.

Gayanilo FC Jr, Sparre P & Pauly D 1996. The FAO ICLARM stock assessment tools (FiSAT) user's guide. FAO Computerized Information Series (Fisheries).

Geary DH 1990. Patterns of evolutionary tempo and mode in the radiation of *Melanopsis* (Gastropoda; Melanopsidae). Paleobiology. 16: 492-511.

Glaubrecht M 1993. Mapping the diversity: Geographical distribution of the freshwater snail Melanopsis (Gastropoda: Cerithioidea: Melanopsidae) with focus in its systematics in the Mediterranean Basin. Mitt. Hamb. Zool. Mus. Inst. 90: 41-97.

Guerra-Merchán A, Serrano F, Garcés M, Gofas S, Esu D, Gliozzi E & Grossi F 2010. Messinian Lago-Mare deposits near the Strait of Gibraltar (Malaga Basin, Southern Spain). Palaeogeogr. Palaeocl. 285: 264–276.

Hartog CD 1974. Brackish-water classification, its development and problems. Hydrobiol. Bull. 8: 15-28.

Heino J, Virkkala R & Toivonen H 2009. Climate change and freshwater biodiversity: detected patterns, future trends and adaptations in northern regions. Biol. Rev. 84: 39–54.

Heller J & Abotbol A 1997. Litter shredding in a desert oasis by the snail *Melanopsis praemorsa*. Hydrobiologia. 344: 65-73.

Hershler R, Liu H & Sada DW 2007. Origin and diversification of the soldier meadow springsnails (Hydrobiidae: *Pyrgulopsis*), a species flock in the Northwestern Great Basin, United States. J. Mollus. Stud. 73: 167–183.

Huey RB & Kingsolver JG 1989. Evolution of thermal sensitivity of ectotherm performance. Trends Ecol. Evol. 13: 299-305.

Liu HP & Hersher R 2012. Phylogeography of ana endangered Western North American springsnail. Conserv. Genet. 13: 299-305.

Lurman G, Walter J & Hoppeler HH 2014. Seasonal changes in the behaviour and respiration physiology of the freshwater duck mussel, *Anodonta anatina*. J. Exp. Biol. 217: 235-243.

Lydeard C, Cowie H, Ponder WF, Bogan AE, Bouchet P, Clark S, Cummings KS, Frest TJ, Gargominy O, Herbert DG, Hershler R, Perez KE, Roth B, Seddon M, Strong EE & Thompson FG 2004. The global decline of nonmarine Mollusks. BioScience. 54: 321-330.

Lysne SJ, Perez KE, Brown KM, Minton RL & Sides JD 2008. A review of freshwater gastropod conservation: challenges and opportunities. J. N. Am. Benthol. Soc. 27: 463–470.

Madsen H 1992. Food selection by freshwater snails in the Gezira irrigation canals, Sudan. Hydrobiologia. 228: 203-217.

Martin AP, Echelle AA, Zegers G, Baker S & Keeler-Foster CL 2012. Dramatic shifts in the gene pool of a managed population of an endangered species may be exacerbated by high genetic load. Conserv. Genet. 13: 349–358.

Martinez MA & Thome DM 2006. Habitat Usage by the Page springsnail, *Pyrgulopsis morrisoni* (Gastropoda: Hydrobiidae), from Central Arizona. Veliger. 48: 8–16.

Mladenka GC & Minshall WG 2001. Variation in the life history and abundance of three populations of Bruneau hot springsnails (*Pyrgulopsis bruneauensis*). West. N. Am. Naturalist. 61: 204–212.

Olden JD, Kennard MJ, Leprieur F, Tedesco PA, Winemiller KO & Garcìa-Berthou E 2010. Conservation biogeography of freshwater fishes: recent progress and future challenges. Divers. Distrib. 16: 496–513.

Por FD 1972. Hydrobiological notes on the high-salinity waters of the Sinai Peninsula. Mar. Biol. 14, 111-119.

Reusch TBH 2014. Climate change in the oceans: evolutionary versus phenotypically plastic responses of marine animals and plants. Evol. Appl. 7: 104–122.

SIRA 2011. Regional Environmental Informative System of Tuscany - http://sira.arpat.toscana.it/sira/bandat.html. Accessed July 2011.

Stewart DJ, Ruffell A, Wach G & Cioldring R 1991. Lagoonal sedimentation and fluctuating salinities in the Vectis Formation (Wealden Group, Lower Cretaceous) of the Isle of Wight, southern England. Sediment. Geol. 72: 117-134.

Strayer DL 2006. Challenges for Freshwater Invertebrate Conservation. J N Am Benthol Soc. 25: 271-287.

Venice System 1959. The final resolution of the symposium on the classification of brackish waters. Arch. Oceanogr. Limnol. 11: 243–248.

Thomsen J, Casties I, Pansch C, Körtzinger A & Melzner F. 2013. Food availability outweighs ocean acidification effects in juvenile *Mytilus edulis*: laboratory and field experiments. Global Change Biol. 19: 1017–1027,

Underwood A, Chapman M & Richards S 2002. GMAV-5 for Windows. An Analysis of Variance Programme. Centre for Research on Ecological Impacts of Coastal Cities. Marine Ecology Laboratories, University of Sydney, Australia.

Vinyard GL 1996. Distribution of a thermal endemic minnow, the desert dace (*Eremichthys acros*), and observations of impacts of water diversion on its population. Great Basin Nat. 56: 360-368.

Watson AM & Ormerod SJ 2004. The distribution of three uncommon freshwater gastropods in the drainage ditches of British grazing marshes. Biol. Conserv. 118: 455–466.

Woodward G, Perkins DM & Brown LE 2010. Climate change and freshwater ecosystems: impacts across multiple levels of organization. Phil. Trans. R. Soc. B. 365: 2093–2106.

1
2
3 ## Tables
4
5 **Table 1** Average (av.), maximum (max), minimum (min) and standard deviation (sd)
6 values of physico-chemical water parameters for upstream (Ups) and extinction
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8 (Ext) positions of the considered streams. Unit of measure for ionic concentrations
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10 is mg/L.

Parameter		Bruna		Aronna		Roselle		Roselle-*Amph*	Venturina	
		Ups	*Ext*	*Ups*	*Ext*	*Ups*	*Ext*		*Ups*	*Ext*
T (°C)	av.	18.3	17.3	20.6	15	35.8	22.2	36.6	33.5	26.6
	max	25.7	25	22.7	21.9	36.5	27.3	38.1	35.2	31.3
	min	13.7	12.9	19	8.5	34.6	18.2	34.6	30	20.1
	ds	5.7	5.7	1.7	7	0.8	4	0.5	2.4	5.6
pH	av.	7.4	7.8	7.4	8	7.3	7.6	-	7.3	7.9
(20 °C)	max	7.6	7.9	7.5	8.4	7.5	7.7	-	7.6	8
	min	7.1	7.7	7.2	7.8	7.2	7.4	-	6.8	7.7
	ds	0.2	0.1	0.1	0.2	0.1	0.1	-	0.4	0.1
TDS	av.	1938	1831	2084	1736	2638	2546		1627	1685
	max	2065	2015	2144	2045	2678	2580		1772	1739
	min	1755	1523	1952	1381	2598	2509		1368	1639
	ds	135	223	89	315	35	30		178	44
Na^+	av.	12	12.8	11.5	13.5	28	31	-	28.5	29.3
	max	13	14	12	17	29.5	34	-	31	31
	min	11	12	11	11	26	29	-	27	28
	ds	0.8	1	0.6	2.6	1.5	2.2	-	1.9	1.5
K^+	av.	3	3	3.8	3	3.5	4.7	-	4.6	4.6
	max	3.2	3.2	6	3.4	4.3	7	-	5	5
	min	2.8	2.7	3	2.4	3.1	3.5	-	4.3	4.3
	ds	0.2	0.2	1.5	0.4	0.5	1.6	-	0.3	0.3
Mg^{2+}	av.	98	93	112	95	122	116	-	80	78
	max	106	101	122	111	125	118	-	83	81
	min	90	78	105	72	118	114	-	78	76
	ds	7	10	7	19	3	2	-	2	2
Ca^{2+}	av.	405	379	442	355	575	556	-	364	352
	max	426	418	465	423	592	566	-	379	365
	min	364	313	407	289	564	545	-	352	341
	ds	29	50	25	74	12	9	-	12	13
NH_4^+	av.	0.04	0.02	0.03	0.06	0.05	0.04	-	0.05	0.02
	max		-		-		-	-		-

Parameter		Bruna		Aronna		Roselle		Roselle-Amph	Venturina	
		Ups	Ext	Ups	Ext	Ups	Ext		Ups	Ext
	min		-		-			-	-		
	ds		-		-			-	-		
F^-	av.	1.1	1.1	1.3	1.2	2.1	2	-	1.5	1.5
	max	1.2	1.2	1.5	1.4	2.4	2	-	1.6	1.8
	min	1	0.9	1.2	1	2	1.9	-	1.4	1.3
	ds	0.1	0.1	0.1	0.2	0.2	0.1	-	0.1	0.2
Cl^-	av.	17.3	17.5	16	25.5	34.9	36.3	-	36.5	39
	max	19	19	18	49	38.5	40	-	40	46
	min	16	16	14	15	32	34	-	33	34
	ds	1.5	1.3	1.6	16	3.1	2.9	-	3.5	6
NO_3^-	av.	2.5	2.2	2.8	2.1	2.8	4.8	-	2.2	4.2
	max	2.8	2.4	3	3.2	3.5	7.9	-	2.5	6.4
	min	1.9	1.7	2.6	1.2	1.8	3.2	-	2	2.6
	ds	0.4	0.4	0.2	0.9	0.7	2.2	-	0.2	1.7
SO_4^{2-}	av,	1156	1094	1238	1063	1609	1554	-	889	969
	max	1217	1199	1289	1271	1664	1586	-	1020	1004
	min	1030	879	1133	721	1572	1510	-	621	937
	ds	88	151	71	256	39	32	-	181	35
HCO_3^-	av,	243	228	257	179	260	242	-	221	209
	max	280	260	268	262	265	257	-	227	234
	min	226	217	242	102	257	232	-	216	183
	ds	25	21	11	81	3	11	-	4	21

Table 2 Results of the two-way multivariate permutational analysis of variance (PERMANOVA) conducted on $\sqrt{(x + 1)}$ transformed physico-chemical water parameters. Factors are: Site ('Si'; four levels: Bruna, Aronna, Venturina, Roselle; orthogonal and fixed, Position ('Po'; two levels: upstream and extinction; orthogonal and fixed). The degrees of freedom, df, variance, MS, value of pseudo-F ratio and probability levels, P, are shown. Number of replicates: 4.

Source	df	SS	MS	pseudo-F	P
Site (Si)	3	215.86	71.95	17.898	0.001
Position (Po)	1	35.61	35.61	4.4437	0.037
SixPo	3	24.04	8.01	1.9933	0.007
Res	24	96.49	4.02		
Total	31	372.00			

Table 3 Results of the two-way analysis of variance (ANOVA) conducted on two main physico-chemical parameters. Factors are: Site ('Si'; four levels: Bruna, Aronna, Venturina, Roselle; orthogonal and fixed, Position ('Po'; two levels: upstream and extinction; orthogonal and fixed). The degrees of freedom, df, variance, MS, value of F ratio and probability levels, P, are shown. Number of replicates: 4.

Source	DF	pH			NO_3^-		
		MS	F	P	MS	F	P
Site (Si)	3	0.0772	2.09	0.128	0.2102	3.71	*0.025*
Po (Po)	1	1.6313	24.79	*0.016*	0.2052	0.85	0.425
SiXPo	3	0.0658	1.78	0.178	0.2419	4.27	*0.015*
RES	24	0.0369			0.0567		
TOT	31						

Table 4 Results of the two-way analysis of variance (ANOVA) conducted on snails density and average shell lengths. Factors are Site ('Si'; four levels: Bruna, Aronna, Venturina, Roselle; orthogonal and fixed, Transect ('Tr'; two levels; random and nested in 'Si'). The degrees of freedom, df, variance, MS, value of F ratio and probability levels, P, are shown. Number of replicates: 8.

Source	df	Density (ind/m^2)			Size (SL)		
		MS	F	P	MS	F	P
Si	3	152254.28	8.1	*0.036*	3.48	11.0	*0.020*
Ti(Si)	4	18847.41	1.0	0.440	0.31	0.8	0.530
Res	24	19337.55			0.38		
Total	31						

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Table 5 Battacharya determinations of *M. etrusca* age cohorts at different streams, based on summer and winter populations' samplings. Computations are based on 0.5 mm shell length intervals. For each age cohort, the mean shell length ('Mean') the relative satandard deviation ('S.D.'), the total number of observations ('n') and the Separtaion Index ('S.I.') are reported. S.I. values smaller than 2 are showed in italics.

Site	Age Cohort	JULY 2009				FEBRUARY 2010			
		Mean	S.D.	n	S.I.	Mean	S.D.	n	S.I.
Bruna	I	2.91	0.71	244	n.a.	1.9	0.56	1036	n.a.
	II	6.01	0.75	130	4.25	4.64	1.71	362	2.41
	III	8.13	0.67	48	2.99	11.1	1.17	46	4.49
	IV	10.87	0.84	57	3.63				
Valpiana	I	4.17	1.48	548	n.a	2.57	0.66	431	n.a
	II	7.85	1.31	77	2.32	4.27	1.14	418	*1.93*
Roselle-*Amph*	I	2.15	0.52	30	n.a.	2.82	0.69	421	n.a
	II	5.63	1.15	81	4.17	6.91	1.66	142	2.99
	III	8.23	1.11	101	2.3	10.99	0.46	23	2.46
Roselle	I	3.17	0.86	1809	n.a.	1.38	0.73	1876	n.a
	II	6.76	0.66	103	4.72	3.91	1.01	217	2.75
	III					6.55	0.71	29	2.37
Venturina	I	3.87	0.86	1987	n.a.	2.9	0.87	2220	n.a.
	II	5.77	0.90	143	2.16				

Table 6 Results of the three-way multivariate permutational analysis of variance (PERMANOVA) carried out on *M. etrusca* shell length increments during the first month of growth experiment. Factors are Site ('Si'; two levels: Bruna and Venturina; orthogonal and fixed), Position ('Po'; two levels: upstream and extinction; orthogonal and fixed), Mesocosm ('Me'; four levels; random and nested in 'Si' and 'Po'). The degrees of freedom, df, variance, MS, value of pseudo-F ratio and probability levels, P, are shown. Number of replicates: 30.

Source	df	SS	MS	pseudo-F	P
Si	1	0.172	0.172	7.587	*0.024*
Po	1	0.973	0.973	42.792	*0.001*
SixPo	1	0.888	0.888	39.077	*0.001*
Me(SixPo)	11	0.281	0.026	1.830	*0.048*
Res	332	4.639	0.014		
Total	346	7.643			

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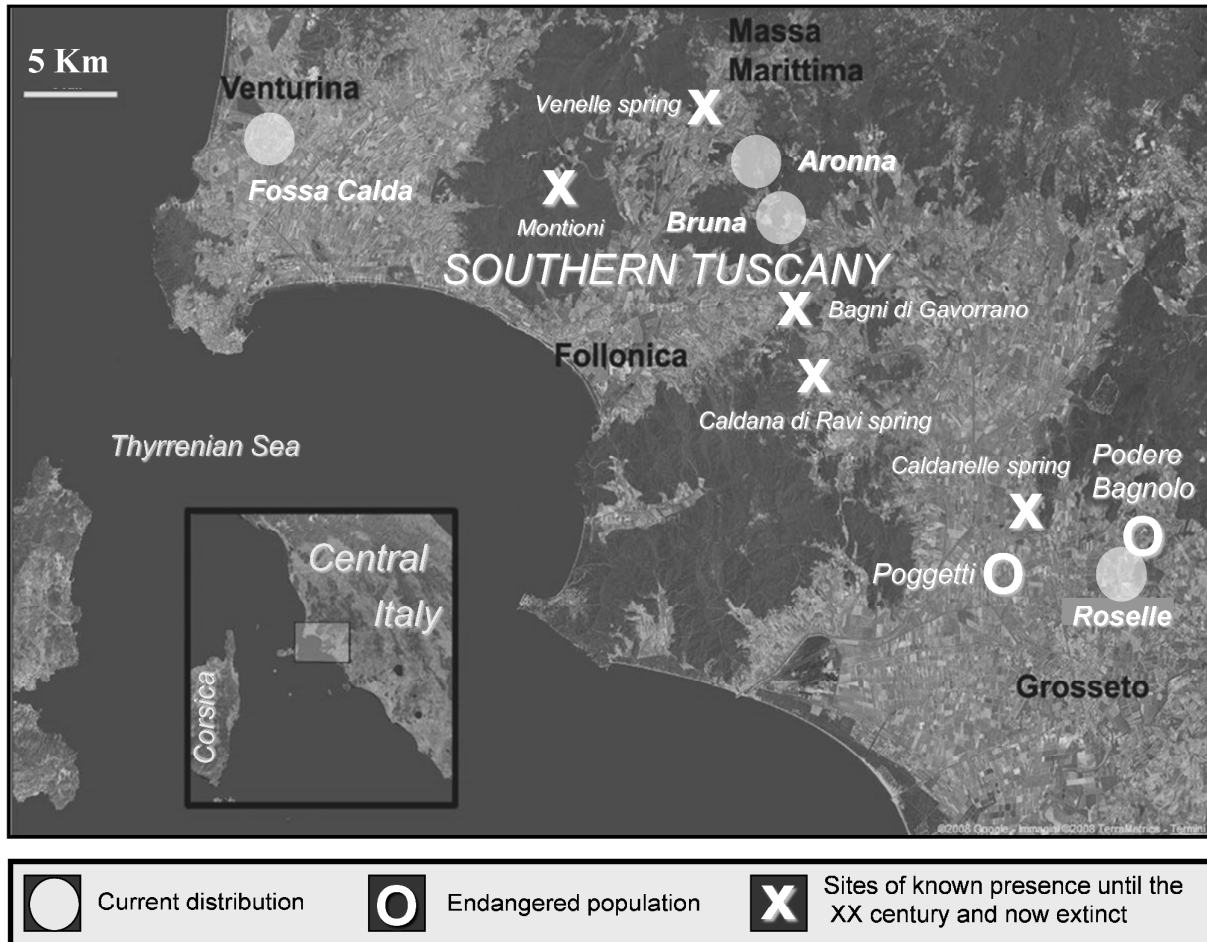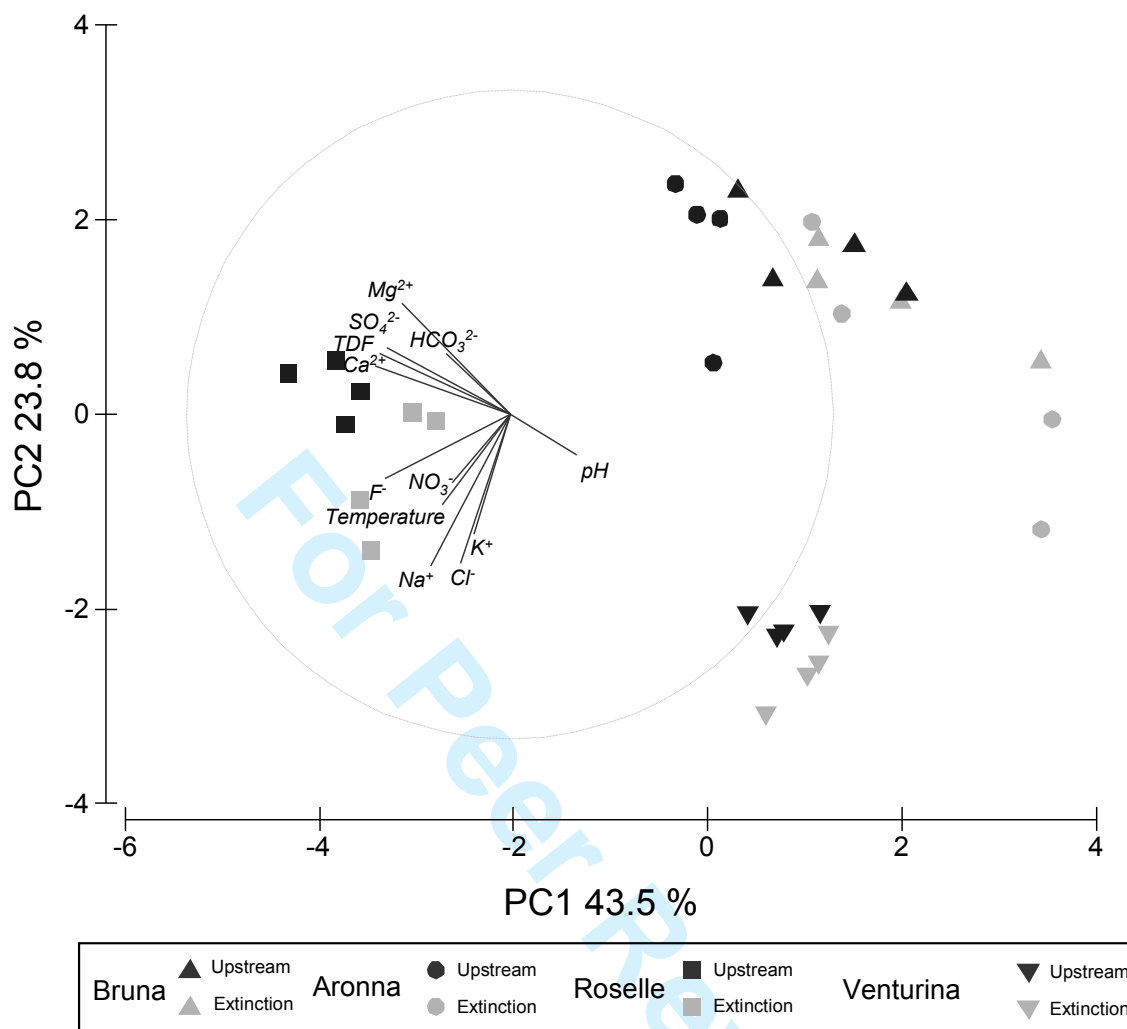52
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Figures

Figure 1 Present and past distribution of *Melanopsis etrusca* in Southern Tuscany, back to early XX century. The species dwells the first few hundred meters of streams fed by thermo-mineral springs.

Figure 2 Two-dimensional scatter plot (PCA) of first and second principal components for physico-chemical water parameters of the four study sites. For each stream, upstream and extinction positions were considered. Vectors of the linear correlations between individual variables are superimposed on the graph.
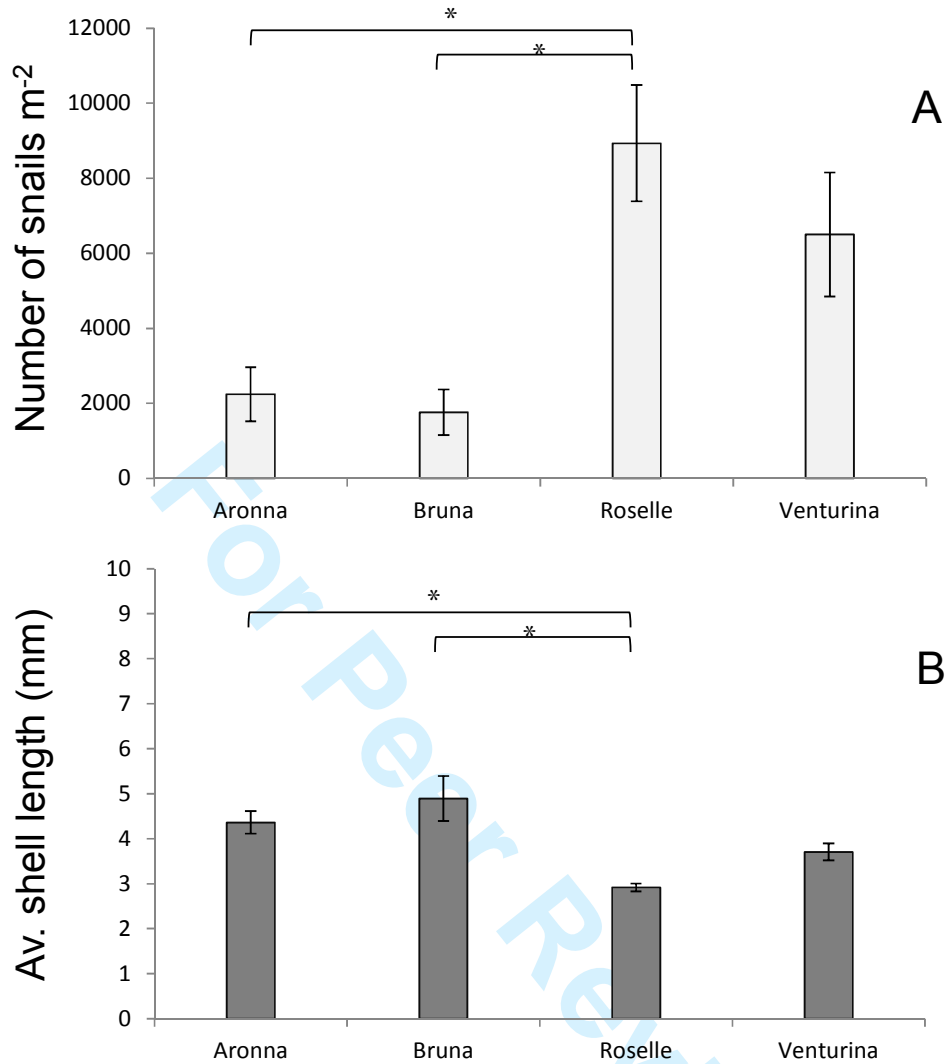
Figure 3 Average values of *M. etrusca* density per square meters (A) and shell length (B) measured at upstream positions of different streams. *: statistically significant differences with a probability level of 95%. Error bars are ± 1 SE.
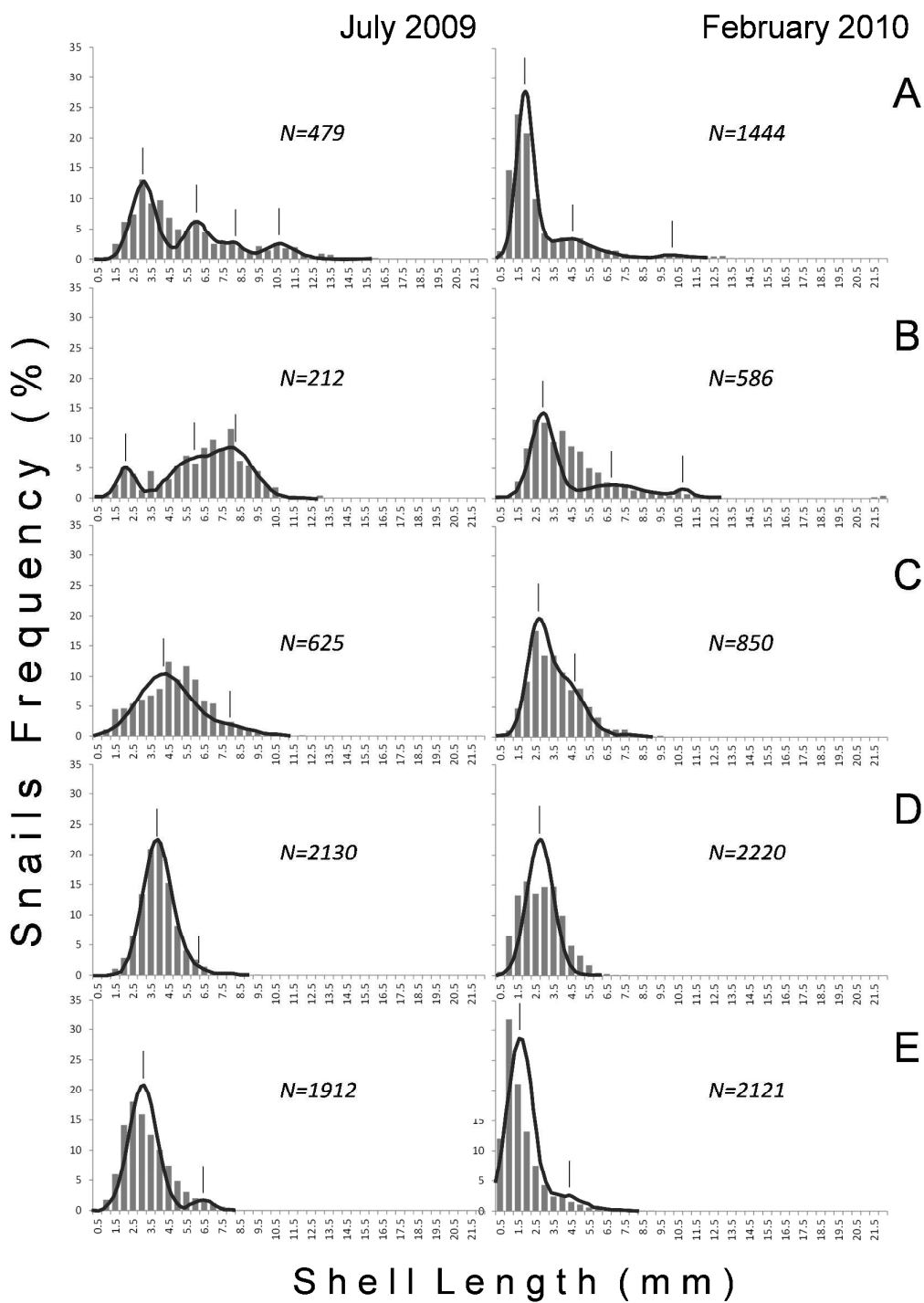
Figure 4 Frequency distribution of *M. etrusca* shell length classes of 0.5 mm at different sites, with superimposed the age cohorts (solid lines and vertical bars) as determined by Battacharya computation. (A: Bruna; B: Roselle-'amphibians'; C: Aronna; D: Venturina; E: Roselle).
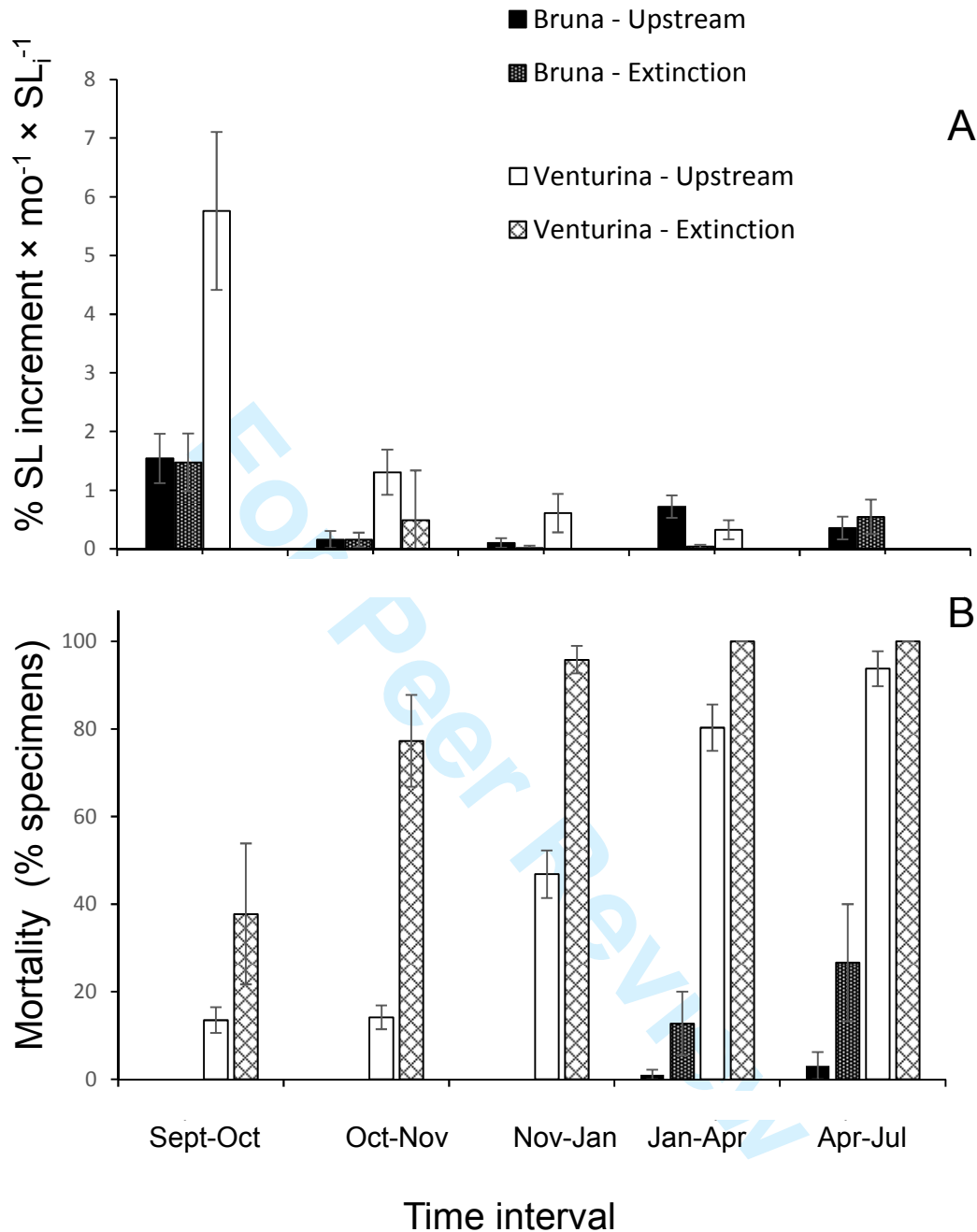
Figure 5 Results of individual growth and mortality inside the mesocosms at Bruna and Venturina, from September 2009 until July 2010. A) Average monthly shell length increments estimated from the considered time intervals. B) Average cumulative percentage mortality. Error bars indicate 99% confidence intervals.
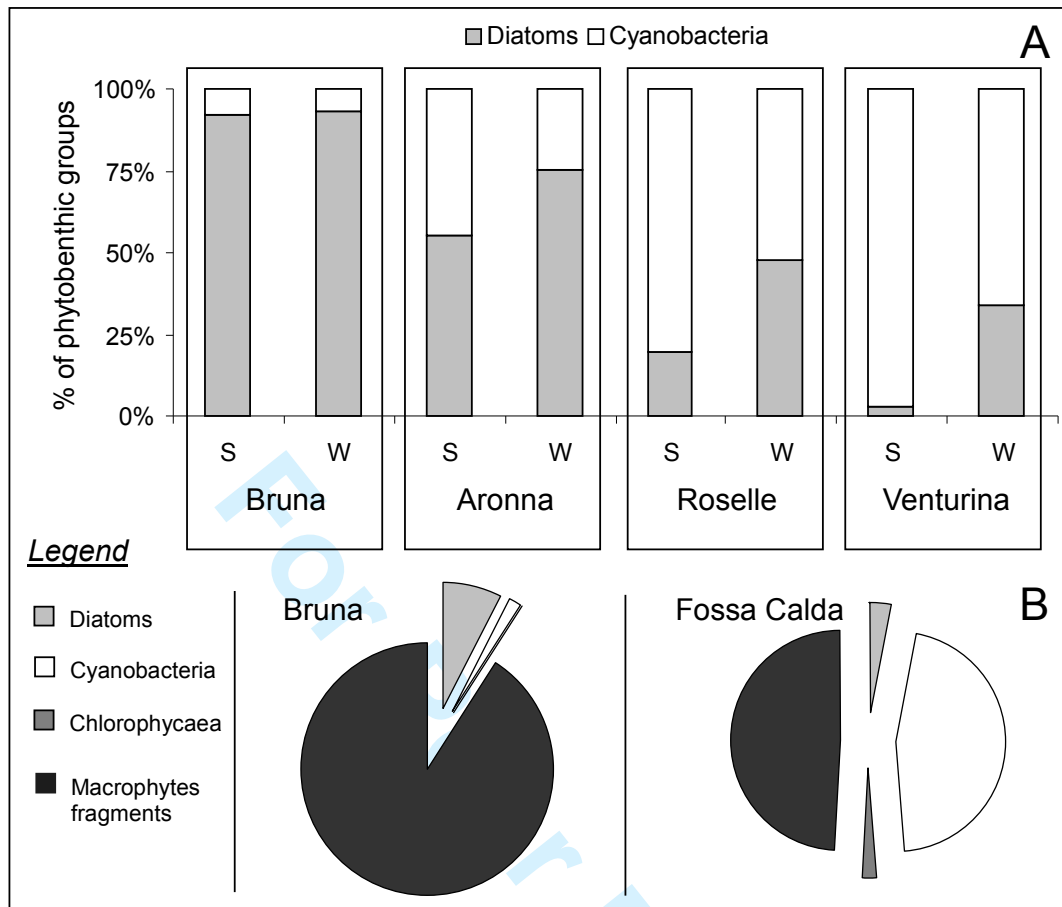
Figure 6 A) Semi-quantitative estimate of summer and winter abundances of phytobenthic organisms found on hard substrates at upstreams position of different streams. B) Relative abundance of main food items found inside the crop of *M. etrusca* specimens (n=10) collected at Bruna and Venturina in summer 2010.