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Molt and gastroliths in *Austropotamobius pallipes* (Lereboullet, 1858)

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ABSTRACT

Key-words: *white-clawed crayfish, Astacidae, ecdysis, molting period, gastrolith size*

Knowledge of the molting cycle in crayfish may contribute (1) to improving a population analysis for monitoring and conservation purposes and (2) to imposing significant constraints on practices involved in culture. Two populations of *Austropotamobius pallipes* in central Italy were monitored from April to November for five years (2002–2006). Crayfish were collected, sexed, and the carapace length (CL) was measured. Molt cycle stages (premolt, molt, postmolt and intermolt) were identified depending on the exoskeleton hardness and color, and the abdomino-thorax splitting. The molt lasted for 6 minutes in juveniles, and maximally 16 minutes in adults in captivity. Males and females were treated separately, and divided into five age classes (0+; 1+; 2+; 3+; >4+). No significant differences were observed between females and males of the same age class. The molting event mainly occurred in April and May ongoing to November. The molting cycle occurred less frequently between July and August, and during the study period it was rarely observed between the last five days of July and the first week of August. Moreover, we measured and counted the gastrolith layers from a total of 124 crayfish (73 females + 51 males) collected twelve years ago, in order to discuss possible correlations between CL and gastrolith size.

RÉSUMÉ

Mue et gastrolithes chez *Austropotamobius pallipes* (Lereboullet, 1858)

Mots-clés : *écrevisse à pattes blanches, Astacidés, ecdysis, période de mue, taille du gastrolithe*

La connaissance du cycle de mue chez l'écrevisse peut contribuer (1) à améliorer l'analyse d'une population à des fins de suivi et de conservation et (2) à imposer des contraintes importantes à des pratiques d'élevage. Deux populations d'*Austropotamobius pallipes* d'Italie centrale ont été suivies d'avril à novembre pendant cinq ans (2002–2006). Les écrevisses ont été collectées, sexées et la longueur de la carapace (CL) mesurée. Les stades du cycle de mue (pré-mue, mue, post-mue et inter-mue) ont été identifiés en fonction de la dureté et de la couleur de l'exosquelette et de la fente abdomino-thoracique. La mue dure 6 minutes chez les juvéniles et au maximum 16 minutes chez l'adulte en captivité. Mâles et femelles ont été examinés séparément, et divisés en cinq classes d'âge (0+ ; 1+ ; 2+ ; 3+ ; >4+). Aucune différence significative n'a été observée entre mâles et femelles d'une même classe d'âge. La mue se produit principalement en avril et mai et se prolonge jusqu'en novembre. La mue se produit moins fréquemment entre

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juillet et août, et pendant la période d'étude elle a été rarement observée pendant les cinq derniers jours de juillet et la première semaine d'août. De plus, nous avons mesuré et compté les couches des gastrolithes sur un total de 124 écrevisses (73 femelles + 51 mâles) collectées 12 ans auparavant, pour rechercher des corrélations éventuelles entre CL et taille du gastrolithe.

INTRODUCTION

The external skeleton is one of the most interesting features of crustaceans, that has permitted their colonization of various types of marine and freshwater habitats. This exoskeleton puts different constraints on crustaceans' growth. In fact, size increase is possible after a periodic shedding of the exoskeleton that permits the expansion of soft tissues and replacement of the cuticle, the last phenomenon being better known as molting or ecdysis. In some crustaceans, molting can take place throughout their life cycle, whereas other species molt only until sexual maturity is reached (Hartnoll, 1982). The preparation for ecdysis is under control of the endocrine system, which responds to internal (physiological) and external (environmental) factors (Aiken and Waddy, 1992).

In crayfish, the molting cycle involves several steps (Aiken and Waddy, 1992; Reynolds, 2002), often summarized in four main stages: premolt (PR), molt (MO), postmolt (PO) and intermolt (IN). During the last (longest) one (Drach and Tchernigovtzeff, 1967), most of the calcium is stored in the cuticle. But during PR, calcium is also stored in paired, hard, thick, disc-like structures known as gastroliths (mainly consisting of an amorphous calcium carbonate different from calcite – Addadi *et al*., 2003; Ahearn *et al.*, 2004), located within cavities on both sides of the stomach wall of the new exoskeleton (Travis and Friberg, 1963). At ecdysis (MO stage), the gastroliths collapse into the stomach lumen where they are chemically and mechanically digested and converted into ionic calcium for use in postmolt mineralization (Travis, 1960; Suko, 1968). Then, rapid remineralization of the cuticle relies on the calcium digested from the gastroliths, together with environmental calcium absorbed from water (Reynolds, 2002).

Ecdysis seems not only to be an important phenomenon for growth but also for the sanitary state of crustaceans. In fact, after molting, crayfish have the opportunity to leave parasites (Gherardi *et al*., 2002; Scalici *et al*., 2010b), so that the latter do not damage integument tissues.

The ecdysis cycle determines the typical stepwise growth pattern of crustaceans, sometimes making studies on the population structure and dynamics hard when non-invasive marking methods are used (Scalici *et al*., 2010a). Although modal progression analysis can be usable for estimating dynamic properties in freshwater decapod populations (Scalici *et al*., 2008, 2008b), sometimes characteristics such as age structure, abundance and density are hardly assessable although widely required in population analyses and/or conservation activities (Scalici *et al*., 2010a). In this case, knowledge of the molting cycle and factors affecting it may contribute to improving a population analysis, and in addition imposes significant constraints on practices involved in crayfish aquaculture.

The main aim of this study is to improve the knowledge of the molting cycle of the white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) in central Italy for monitoring and conservation purposes. Additionally, we studied the gastroliths in order to verify whether their structure can help in population studies, particularly in determination of the crayfish age.

MATERIALS AND METHODS

> *COLLECTION OF DATA AND ANALYTICAL PROCEDURE*

Crayfish were caught by hand-nets and traps from April to November during the period 2002–2006 in two close together sites, at a similar latitude and altitude (central Italy – Figure 1).

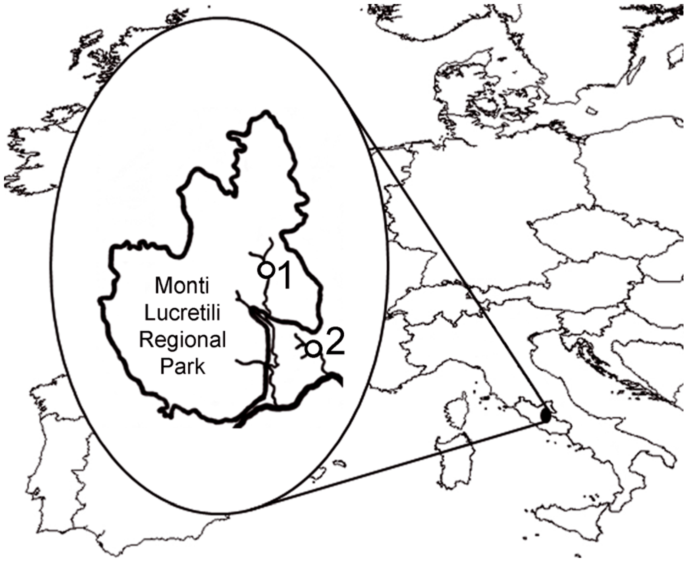

Figure 1
Sampling locations within the study area (for more geographic information see Scalici et al., 2008): 1, River Licenza; 2, River Duranna.

Figure 1
Sites échantillonnés dans l'aire d'étude (pour plus d'informations géographiques voir Scalici *et al*., 2008) : 1, Rivière Licenza ; 2, Rivière Duranna.

Crayfish were sexed, and the carapace length (CL – from the ocular hollow to the terminal thorax portion) was measured. The main four molting cycle stages were identified depending on the exoskeleton hardness and color, and the existence of abdomino-thorax split. During each sampling, entire exuviae were also recorded, sexed, the length of the carapace was taken, and then exuviae were shattered in order to avoid duplication of measurements and records on the same exuvia on the next sampling occasion. Carapaces and parts of exuviae were not recorded since they were considered as a drift.

Additionally, 124 crayfish (73 females + 51 males) ranging from 7 to 33 mm CL were collected within the same study area during an entire calendar year twelve years ago, preserved in absolute ethanol, and selected for morphometric and microscopic analyses of the gastroliths. In particular, both height (GH) and diameter (GD) were surveyed only for the right gastrolith (since they are symmetric structures), and the left one of the same specimen was prepared for a scanning electron microscope (SEM) by rinsing in water and drying in air. Then dry samples were dipped in liquid N_2, broken, and gold-sputtered according to Shechter *et al*. (2008), in order to count the number of the gastrolith layers (GL), *i.e.* concentric layers of amorphous calcium carbonate intercalated between chitinous lamellae (Shechter *et al*., 2008).

Finally, captivity observations were made, using animals collected from the same study area with the aim of detecting the exact molting time. In total, 50 crayfish were reared in a recirculating system, 10 (5 females + 5 males) per the following age classes (selection was made according to CL – for more details see Scalici *et al*., 2008): 0+ (first life year); 1+ (second); 2+ (third); 3+ (fourth); $>$4+ ($>$ fifth). They were fed *ad libitum* and the water temperature simulated the temperature trend of the water in the study area (obtained during each sampling by an immersion probe) (Scalici and Gibertini, to appear).

> *STATISTICAL ANALYSIS AND SOFTWARE*

The χ^2 test was performed to verify differences in the molting period between (1) juveniles (individuals with CL $<$ 23 and 27 for females and males, respectively – Scalici and Gibertini,

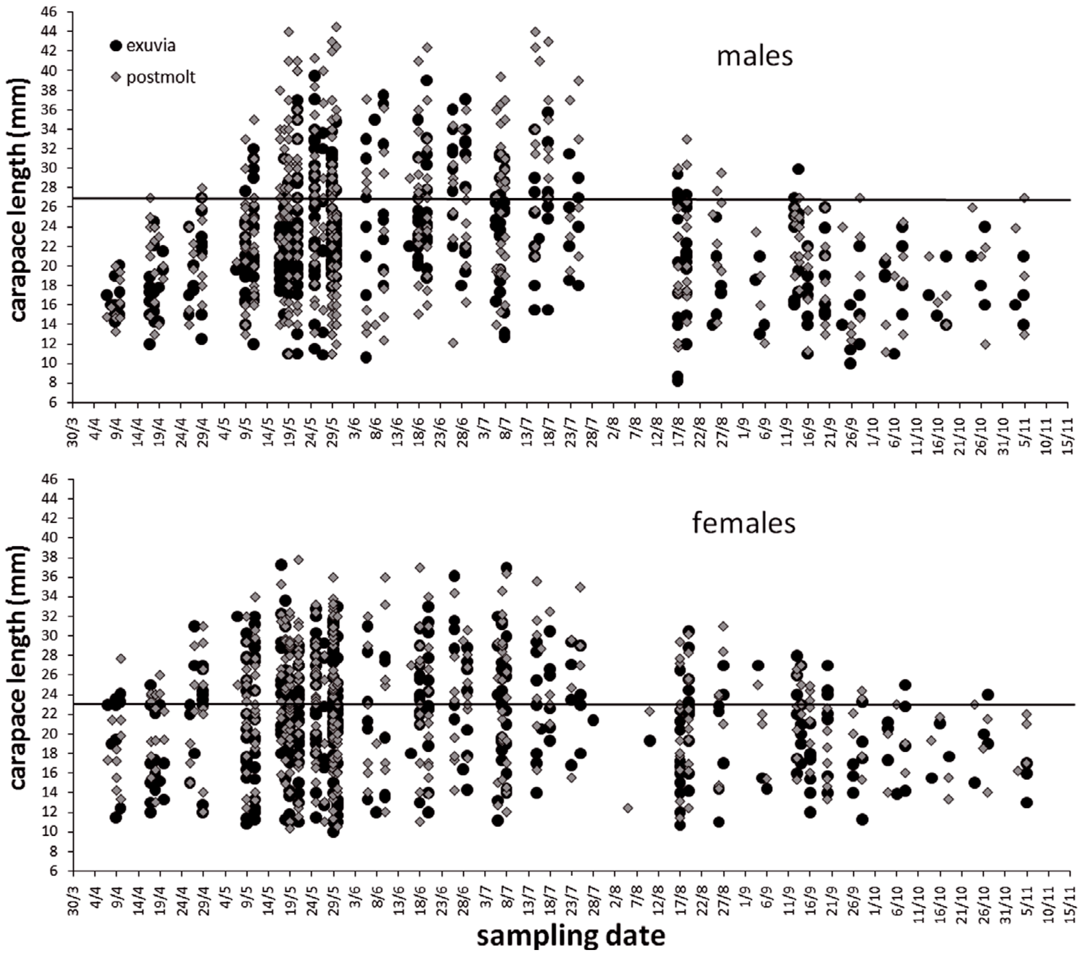

Figure 2
Postmolt individuals and exuviae per size collected throughout the study years, both for males and females. Black lines indicate the carapace length at maturity.

Figure 2
Individus en post-mue et taille de l'exuvie collectée pendant toutes les années de l'étude, à la fois pour les mâles et les femelles. Les lignes noires indiquent la longueur de la carapace à la maturité.

to appear) and adults, and (2) sexes. In this case the observed frequency of the PO individuals and the exuviae was used separately. Next, three regression analyses were performed for the sexes separately: "GH *vs.* CL"; "GD *vs.* CL"; and "GL *vs.* CL". Difference in slope of the regression function between females and males was tested by analysis of covariance (ANCOVA) with size as a covariate. Finally, differences in the molting time (1) between sexes of each age class and (2) among age classes were verified by a series of pairwise Kruskall-Wallis tests.
All statistical tests were performed using SPSS 16.0.

RESULTS

Molting mainly occurred in April and May ongoing to November for both sexes. It occurred less frequently between July and August, rarely between the last five days of July and the first week of August. Additionally, adult crayfish seemed to have a narrower molting period than juveniles (Figure 2). In particular, a total of 841 (397 females + 444 males) PO individuals and

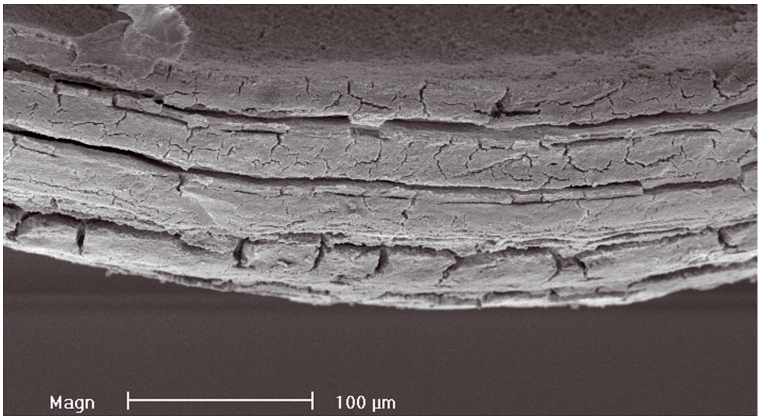

Figure 3
The gastrolith layers observed on the scanning electron microscope (Magn = magnification).

Figure 3
Les couches du gastrolithe observées au microscope électronique à balayage (Magn = grossissement).

863 (419 + 444) exuviae were collected during the entire study period. No significant differences in molting period frequencies were observed between sexes, either in PO specimens (χ^2 = 10.4, df = 7, ns) or exuviae (χ^2 = 12.7, df = 7, ns). Also, no differences were observed between juvenile females and males, either in PO specimens (χ^2 = 8.8, df = 7, ns) or in exuviae (χ^2 = 10.1, df = 7, ns). On the contrary, significant differences were observed between adult females and males, both in PO specimens (χ^2 = 22.5, df = 7, $P < 0.01$) and exuviae (χ^2 = 24.6, df = 7, $P < 0.01$). Further differences in the molting period were significant between juveniles and adults, both in female PO specimens (χ^2 = 15.7, df = 7, $P < 0.05$) and exuviae (χ^2 = 18.4, df = 7, $P < 0.05$), and male PO specimens (χ^2 = 22.3, df = 7, $P < 0.01$) and exuviae (χ^2 = 20.7, df = 7, $P < 0.01$).

After dissection different types of gastroliths were observed: PR gastroliths showed different sizes and were included within the lateral stomach sacs; MO gastroliths were within the stomach lumen and showed a compact and regular structure; PO gastroliths were within the stomach lumen and showed evident signs of digestion.

Only PO gastroliths were used for the analysis of the gastrolith layer (GL) by SEM (Figure 3) and for the regressions *vs.* CL (Figure 4). The GL number was also regressed against CL (Figure 5). All the regressions were performed separately for both sexes. The regressions “GH *vs.* CL” and “GD *vs.* CL” showed a low significance in both cases for both females and males, while “GL *vs.* CL” showed a low significance only for males. Differences in the slope of the regression function between females and males were not significant either for “GD *vs.* CL” ($F_{[1,120]}$ = 3.25, P = 0.9) or for “GH *vs.* CL” ($F_{[1,120]}$ = 2.53, P = 1.3). As concerns “GL *vs.* CL”, analysis of covariance was not performed since female data did not have a significant regression.

The duration of molt for both sexes of different age classes is shown in Figure 6. Molt went on for 6 minutes in juveniles to a maximum of 16 minutes in adults. After a series of pairwise Kruskall-Wallis tests, no significant differences were observed between females and males of each age class, or between the classes 3+ and >4+ for both sexes. All the remaining comparisons showed significant differences (Table I).

DISCUSSION

The ecdysis frequency in the studied populations covered all the study period (from April to November). But crayfish of different size did not molt at the same time. In fact, molt frequency

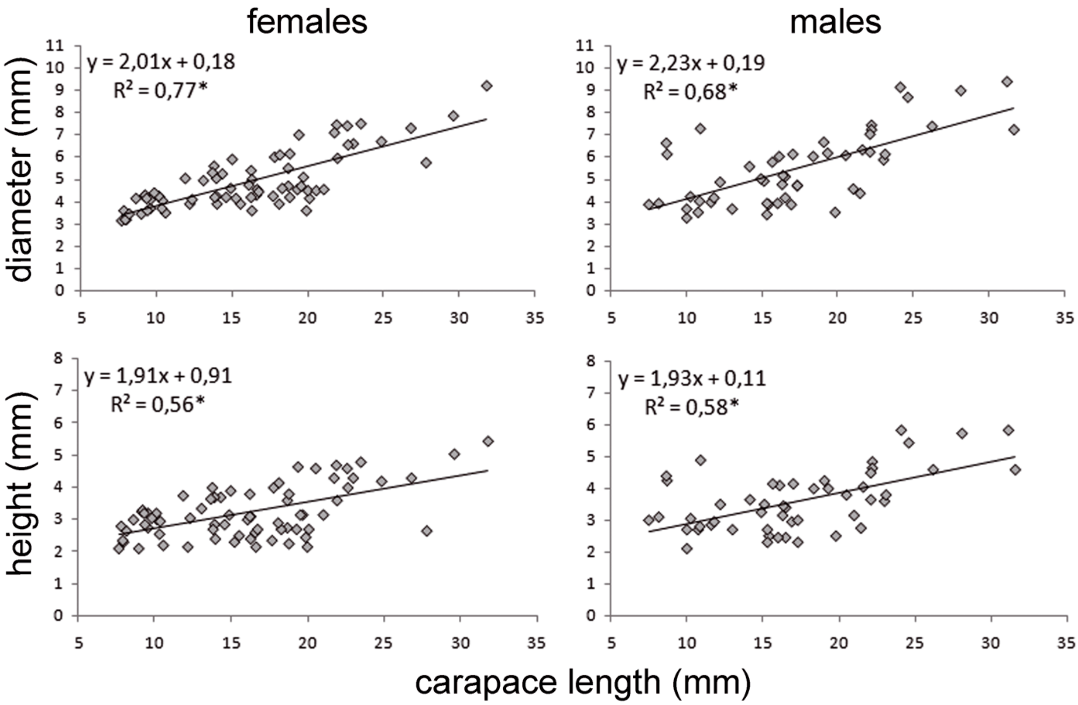

Figure 4
*Relation between height and diameter of right gastroliths and carapace length for both females and males with a regression equation. *P* $<$ 0.05.

Figure 4
Relation entre la hauteur et le diamètre des gastrolithes droits et la longueur de la carapace pour les femelles et les mâles avec l'équation de la droite de régression. **P* $<$ 0,05.

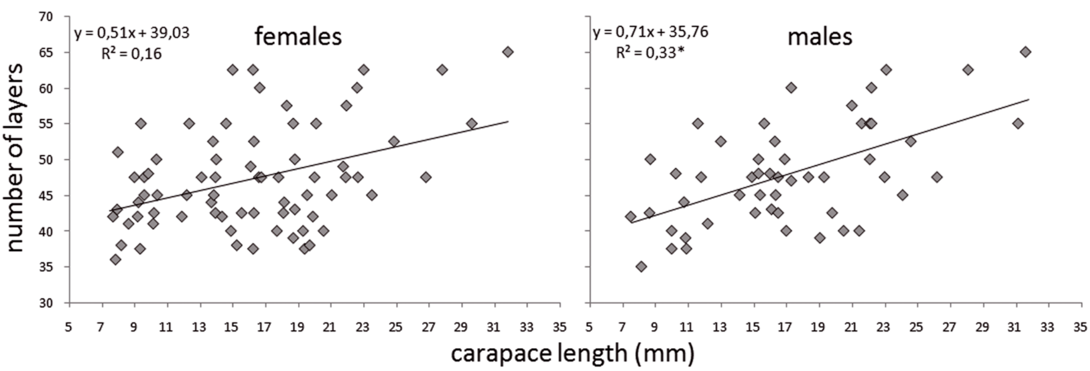

Figure 5
*Relation between number of gastrolith layers and carapace length for both females and males with a regression equation. *P* $<$ 0.05.

Figure 5
Relation entre le nombre de couches du gastrolithe et la longueur de la carapace chez les femelles et les mâles avec l'équation de la droite de régression. **P* $<$ 0,05.

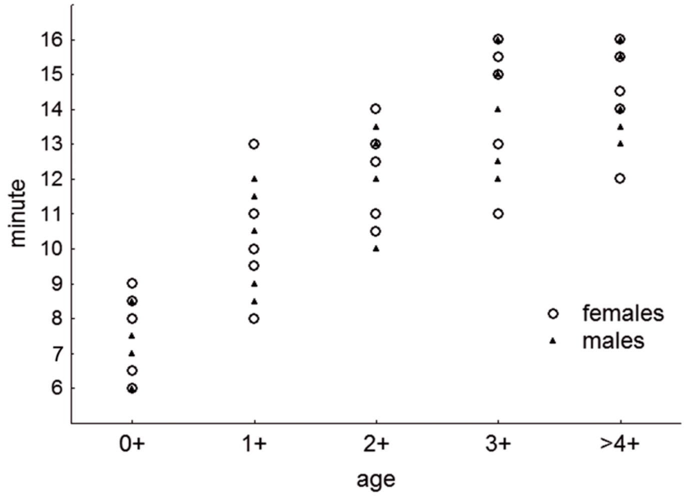

Figure 6
Values of the molting time of the reared individuals for each sex and age cohort.

Figure 6
Durée de la mue d'individus élevés en captivité pour chaque sexe et âge.

Table I
Results of the series of pairwise Kruskall-Wallis tests. Significant values are reported in italics (P is always > 0.05). 0+ = first life year; 1+ = second life year; 2+ = third life year; 3+ = fourth life year; >4+ = fifth life year; F = females; M = males.

Tableau I
Résultats de la série de tests appariés de Kruskall-Wallis. Les valeurs significativement différentes sont en italiques (*P* toujours > 0,05). 0+ = première année de vie ; 1+ = seconde année de vie ; 2+ = troisième année de vie ; 3+ = quatrième année de vie ; >4+ = cinquième année de vie ; F = femelles ; M = mâles.

	F1+	F2+	F3+	F>4+	M0+	M1+	M2+	M3+	M>4+
F0+	*8.48*	*12.43*	*15.11*	*19.74*	4.12	*8.24*	*13.12*	*16.47*	*18.87*
F1+		*13.23*	*18.23*	*19.15*	*8.08*	5.56	*9.53*	*15.57*	*17.49*
F2+			*11.54*	*17.03*	*14.75*	*10.16*	6.33	*12.55*	*16.06*
F3+				3.84	*17.17*	*14.45*	*13.24*	5.91	4.45
F>4+					*20.23*	*19.19*	*17.14*	5.87	6.24
M0+						*9.74*	*13.51*	*16.77*	*18.82*
M1+							*10.33*	*15.24*	*18.43*
M2+								*12.53*	*14.34*
M3+									4.62

and size increase at molt can be differentially affected by the physiological state of the animal and the environmental conditions it encounters (Hartnoll, 1982).

In this study, juveniles molted during all of the study period, except in part of August, whilst adults mainly molted between May and July, although adult females show a wider molting period than adult males (Figure 2). The differences in the molt number between juveniles and adults are due to the different growth rate: after reaching maturity size, adult crayfish have a lower growth rate than juveniles (Scalici *et al*., 2008a). After maturity, the two sexes take two different growth trajectories (Scalici *et al*., 2008a, 2010a), since females spend more metabolic energy on reproduction. But the different growth patterns of the two sexes together with parental responsibility does not affect (narrow) the molting period, as expected. In fact, at different (higher) latitudes adult females and males go through molts at different times (Brewis and Bowler, 1982) and females release their young early in July (S. Peay, pers. comm. in Scalici *et al*., 2008a). On the contrary, in central Italy, adult females release their young in May and molt for a longer period than males, the latter having a bigger percentage of CL increment anyway than the former (Scalici *et al*., 2008a). Additionally, we never observed (except only a few cases) PO individuals and exuviae between the end of July and the beginning of August. This can provide a threefold advantage: (1) this makes the use of non-invasive recapture methods easy during that period for estimating the population size, avoiding the loss of data due to the molt; (2) it reduces the stress for the studied populations; and (3) it also allows one to obtain information about the 'young of the year' since the end of July and the beginning of August coincides with the recruitment period in central Italy (Scalici *et al*., 2008a). This can contribute to studies on the population structure and/or to better stock identification (Cadrin *et al*., 2005).

An upgrading for a better description of the population structure is needed in order also to define demographic properties. In this study we tried to age individuals by the analysis of the gastrolith size and layer organization (*i.e.* number of layers). Unfortunately, it is not possible to evaluate the crayfish age using gastroliths due to the low value of the regression indices, although there are some significant relationships between CL and the surveyed gastrolith features. This means that there is a great overlap amongst cohorts, and gastroliths, although their sizes may be used as a molt state indicator (Pavey and Fielder, 1990), can provide no detailed information on crayfish size, age, or population structure.

Knowledge of the molting period and population structure in crayfish can have useful implications. By using external tags or similar markings in molting periods of less frequency, it is possible to avoid the use of invasive techniques for 'structuring' (*i.e.* ageing) crayfish populations, such as the analysis of lipofuscin granules (Belchier *et al*., 1998), and the recapture method of Abrahamsson (1965) and its modifications (Burič *et al*., 2008; Scalici *et al.*, 2010a), the latter representing the beginning of possible infections that can compromise the health of marked individuals. Invasive methods concern heat-marking on the coxae or carapace by soldering guns and the use of (1) passive integrated transponder tags (Wiles and Guan, 1993; Bubb *et al*., 2002), or (2) visible elastomer tags (Partanen and Penttinen, 1995; Jerry *et al*., 2001). Moreover, beyond the invasiveness, they can be used only for large specimens, since the tools are too large to be used for small individuals. On the contrary, external marks can be used for crayfish of every size.

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