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Original article

# Relictual physiological ecology in the threatened land snail *Codringtonia helenae*: A cause for decline in a changing environment?

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ABSTRACT

Land snails often exhibit intra-annual cycles of activity interspersed by periods of dormancy (hibernation/aestivation), accompanied by a range of behavioural and physiological adaptations to ensure their survival under adverse environmental conditions. These adaptations are useful to understand species-specific habitat requirements and to predict their response to environmental changes. We examined the seasonal physiological and biochemical composition patterns of the threatened land snail *Codringtonia helenae*, endemic to Greece, in relation to its behavioural ecology and climatic conditions. Fuel reserves (glycogen, lipids, proteins) and water were accumulated prior to aestivation, but subsequently were rapidly depleted. LDH exhibited substantial rise during aestivation, suggesting that anaerobic pathways may provide additional energy. The major outcome of our study was the unambiguous discrimination of the four life-cycle periods. Most remarkable was the clear distinction of the aestivation period, with hibernation, the other dormancy period, showing similarity with the two active periods but not with aestivation. We observed disassociation between behavioural and physiological responses and climatic conditions. The physiological responses of *C. helenae* were effective during hibernation, but only partly compensate the effect of adverse conditions during aestivation, since its aestivating behaviour is occasional and time limited. Perhaps, the behavioural ecology of *Codringtonia* is relictual and shaped during past environmental conditions. This constitutes an important extinction threat considering the current climatic trends and the deterioration of the habitat of that species due to human activities.



## 1. Introduction

Land snails in temperate regions often exhibit intra-annual cycles of activity interspersed by periods of dormancy (hibernation/aestivation), accompanied by a range of behavioural and physiological adaptations to ensure their survival under adverse environmental conditions (Cook, 2001). Seasonal variations in land snail behaviour and physiology have been linked to annual cycles of photoperiod, temperature, humidity and water availability (Machin, 1975; Riddle, 1983; Prior, 1985;

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Cook, 2001; Storey, 2002), and can be useful in understanding species-specific habitat requirements, and in predicting their response to future environmental changes. Furthermore, the use of land snails as indicators of the global warming effect on biological processes has promise since the biology of these animals is strongly linked to climate (Goodfriend, 1992; Malcolm et al., 2006), especially in the climatically unpredictable Mediterranean-type ecosystems (Blondel and Aronson, 1999).

Comparative long-term studies on the physiological ecology of land snails, and particularly of those species that show declining populations, may be a useful approach to elucidating environmental-change effects (Cook, 2001; Michaelidis et al., 2007). However, so far, such studies are limited to a few widespread or ''popular'' taxa under laboratory conditions (Herreid, 1977; Wieser and Wright, 1979; Rees and Hand, 1993; Withers et al., 1997) and few studies deal with land snails of the Mediterranean-type ecosystems (e.g. Yom-Tov, 1971b; Warburg, 1972; Brooks and Storey, 1990; Arad et al., 1992, 1995, 1998; Arad and Avivi, 1998; Arad, 1993, 2001; Giokas, et al. 2005; Michaelidis et al., 2007).

The rock snail genus *Codringtonia* Kobelt, 1898 is endemic to Greece. Its member species are listed in the IUCN Red List of Threatened Animals (IUCN, 2006) because populations are in serious decline. In a recent biosystematic revision Subai (2005) showed that the genus includes nine species with interesting distributional patterns that follow a temperature–humidity environmental cline from northwest to southeast continental Greece. *Codringtonia* species are found at various altitudes, living in crevices on rocky terrain within maquis and coniferous (except pines) or mixed (deciduous–coniferous) forests. The only, yet valuable, information about the ecology and life-history of *Codringtonia* comes from an unpublished PhD thesis (Hadjicharalambous, 1996). From that work we know that all species of the genus have a similar biological cycle, albeit with some slight variations due to climatic conditions. Interestingly, *Codringtonia* reproduce in spring, a phenomenon unusual for land snails of southern Europe (Cook, 2001). Furthermore, these snails have two dormancy periods in each annual cycle: a typical long-term hibernation during winter, and a short, occasional aestivation during summer. This uncommon life-history pattern is in disassociation with the prevailing climatic conditions in southern Greece, and warrants further study.

We intend to use *Codringtonia* as a model for ecological, physiological, and phylogenetic studies, in order to infer evolutionary processes and physiological adaptations in the semi-arid Mediterranean region, and as an indicator of the effect of climatic change in that area. This paper reports on the first phase of that project, focusing on the seasonal physiological responses of a particular *Codringtonia* species. This study will serve as a basis for the future comparative studies on the ecophysiology of all *Codringtonia* species. We examined, over one year, sequential monthly samples of adult specimens of *Codringtonia helenae* Subai, 2005 for changes in content of water, energy reserves and lactate dehydrogenase (LDH) in tissues, and related variation to climatic and life-history data. The aims of this study were to: (i) document any seasonal physiological patterns, (ii) discriminate physiological periods based on biochemical variables, (iii) elucidate correlations among physiological, ecological and behavioural responses, and (iv) determine if these responses are adaptive for long-term persistence in the semi-arid environment of southern Greece.

## 2. Materials and methods

### 2.1. The population studied

*Codringtonia helenae* is associated with crevices on limestone substrates that are vegetated by cryptogams such as lichens (the main food of *Codringtonia*), algae and mosses. *Codringtonia helenae* is a large snail (45–54 mm shell diameter in adults). The studied population has a low density ($0.048 \pm 0.034$ specimens/m$^2$) (Hadjicharalambous, 1996). They mate in spring (April) and lay eggs in ground holes (mean $37 \pm 0.99$ per clutch) within 1 month after mating. Eggs are hatched after 30 days (Hadjicharalambous, 1996). Afterwards, both hatchlings and older snails go into aestivation during summer (from July to early September). There is another dormancy period during winter (from November to March). During dormancy successive mucus layers at the shell aperture form the epiphragm as a mechanism for reducing moisture loss (Cook, 2001). In nature, the development from juvenile to fully grown snail takes 4–5 years. The estimated life-span is about 18 years and individual snails contribute to recruitment over several breeding seasons (Hadjicharalambous, 1996).

### 2.2. The study area

The study area was near the village Nestani (about 15 km northeast from Tripolis, 22°23′59″ E, 37°31′58″ N, 800 m a.s.l.). The area occurs in what is known as the mild meso-Mediterranean bioclimatic zone (Mavrommatis, 1978), characterised by 40–75 biologically arid days per annum and 0–3 °C mean minimum temperature of the coldest month. Large blocks of limestone, interspersed with occasional small limestone outcrops and rubble, occur in sites primarily vegetated by maquis (perennial sclerophyllous scrubs of tough shrubby trees; Blondel and Aronson, 1999). Only the large limestone blocks with large crevices provide suitable habitat for *C. helenae*.

### 2.3. Sampling

Random samples (10 specimens) of adult *C. helenae* individuals (i.e. fully grown with reflected/thickened aperture peristome) were taken every month from September 2003 to August 2004, always on non-rainy days, during the last week of each month, at approximately 10.00 am. Using a quadrat count method (see Giokas and Mylonas, 2002) we recorded numbers of adult and juvenile snails and their state of activity (dormant or active) each month. Monthly visits (without sampling) were continued until December 2004 in order to have replicated field observations.

### 2.4. Biochemical and water content analyses

For each month, individuals of *C. helenae* were brought alive to the laboratory within 3 h. They were immediately dissected (after removing the shell) to obtain samples of tissues

principally involved in glycogen storage in land snails (Goddard and Martin, 1996), namely the genitalia, foot muscle, and the hepatopancreas. Tissues from each animal were immediately frozen in liquid $N_2$ so that the metabolites would be preserved, and then were stored at −80 °C. In total, we used tissues from 62 individuals for the biochemical analyses, and from 27 individuals for the water content analyses.

Glycogen (0.1–0.12 g) was determined against a glucose standard by the indirect method of Seifter et al. (1950) after modifying the homogenization procedure according to Pafilis et al. (2005). Measurements were read at 620 nm, using a spectrophotometer (Novaspec II, Pharmacia Biotech).

Total lipids were extracted by homogenizing tissues (30–40 mg) with 1.5 ml of a mixture containing 2 volumes of chloroform and 1 volume of absolute methanol. The homogenate was then centrifuged at 4 °C and 3000 rpm for 10 min. The pellet was used for protein analysis (see below), and the supernatant was used for the determination of total lipid concentration, using the appropriate kit (Chromatest) according to the method of Alexis et al. (1985). A mixture of olive oil and corn oil (2:1 v/v) was used as the standard.

For the determination of total protein levels we used the Biuret method (Layne, 1957). The centrifugation pellet obtained from the lipid analysis (see above) was dissolved in 0.5 ml of 0.1 N NaOH and incubated at 37 °C for 30 min with occasional vortexing. We diluted 50 μl of the sample with 950 μl of $H_2O$ and added 4 ml of the Biuret reagent. The mixture was incubated for 30 min at room temperature and the absorbance was read at 550 nm using a spectrophotometer (Novaspec II, Pharmacia Biotech). Bovine serum albumin (0.5–10 mg/ml) was used as a standard.

Lactate dehydrogenase (LDH) is an enzyme associated with anaerobic pathways, catalyzing the inter-conversion of pyruvate and lactate. We measured LDH activity following the method of Kornberg (1955). We homogenized 0.1–0.15 g of tissue sample, at a ratio of 1:10, with 0.1 M Tris–HCl, pH 7.5, 1 mM EDTA solution. The homogenate was centrifuged at 4 °C and 12,000 rpm for 1 min. The reaction mixture (3 ml) contained 0.1 M Tris–HCl pH 7.3, NADH 0.1% (w/v), 0.15 M pyruvate acid, 6 mM KCN and double distilled water. The reaction was initiated with the addition of 50 μl of the tissue homogenate; we then measured the decrease in absorption at 340 nm, in relation to time. The activity of LDH was calculated using the following formula: $\Delta OD \times 3.05 \times$ dilution (the dilution in Codringtonia's tissues was 1/2).

For the determination of water content, fresh tissues (from the sample of the non-frozen specimens) from each individual were initially weighed on a precision balance and the fresh mass was recorded. The tissues were then placed in an oast-oven at 42 °C and weighted every 12 h until stabilization of mass. We expressed water content as percentage of dry weight [(fresh mass − dry mass)/dry mass] × 100.

### 2.5. *Data analysis*

Based on our field observations that were concordant with bibliographic data (Hadjicharalambous, 1996) we pre-defined four periods: (i) 'aestivation' (samples from July to August), (ii) 'autumn active' (samples from September to October), (iii) 'hibernation' (samples from November to March), and (iv) 'spring active' (samples from April to June). We examined the effect of collection date (month and period) and tissue type (foot muscle, hepatopancreas, genitalia) on the measured biochemical parameters (lipids, glycogen, proteins, LDH) and on water content. We analysed all variables, except water content, simultaneously with multivariate analyses of variance (MANOVA), followed by univariate analyses (one- and two-factor ANOVA). When necessary to meet assumptions of normality of variances, data were log or angular transformed.

In order to identify relations between pairs of mean monthly measures and possible time-delayed effects we used correlation analysis and analyses of time series cross-correlations among monthly estimates.

We used discriminant function analysis (DFA) in order to determine which of the measured variables account the most for the differences between the four predefined periods or between months, and thus examine the adequacy of our a priori assignment to periods. For the perceptual mapping of months we used the discriminant scores of DFA for each month. Moreover, we analysed the structure of interrelationships among the measured variables using principal components analysis (PCA).

Climate data available for the study area (obtained by the Hellenic National Meteorological Service, http://www.hnms.gr), included ambient temperature (mean, maximum, minimum), precipitation, relative humidity and the number of rainy days. We used PCA to analyse the climatic data and to produce a component variable from them. Then, we examined the correlation (parametric and non-parametric) of the variables measured with this climatic component variable. All statistical methods used are described in Zar (1984) and Hair et al. (1998).

## 3. Results

### 3.1. *Discrimination among periods*

DFA revealed an unambiguous discrimination among the four periods (100% of the cases were correctly classified) (Table 1, and Fig. 1a). From the minimum squared Mahalanobis distances between groups (Table 1) and from Fig. 1a it is clear that the aestivation period is well distinguished from the other periods for the variables measured. Hibernation period was placed in between the two active periods. LDH (in all tissues) accounted for most of the differences between aestivation and all the other periods.

The results of the PCA analysis are shown in Table 2 and in Fig. 1b. Components 1, 2 and 3, accounted for 92.18% of variation. LDH (in all tissues), and proteins (in foot muscle and hepatopancreas tissues) contributed most to Component 1, lipids (in all tissues) to Component 2, and glycogen (in all tissues) to Component 3 (Table 3). The plot of the factor scores for Components 1 and 2 shown in Fig. 1b is almost identical with the discriminant function plot, noting that PCA tends to provide the maximum discrimination between individuals, while DFA between predefined groups (Hair et al., 1998). Along the first component (PC1) there is a clear distinction between the aestivation months and all hibernation winter months. In

**Table 1 – Results of the discriminant function analysis (DFA) on the biochemical variables: (A) function matrix showing the contribution of each variable to the three discrimination functions; (B) minimum squared Mahalanobis distances between groups (periods)**

| | Function | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| (A) *Function matrix* | | | |
| Glycogen-foot muscle | -0.117 | 0.091 | -0.008 |
| Glycogen-hepatopancreas | -.0112 | 0.115 | -0.078 |
| Glycogen-genitalia | -0.112 | 0.098 | 0.075 |
| Lipids-foot muscle | -0.173 | 0.431 | 0.214 |
| Lipids-hepatopancreas | -0.085 | 0.251 | 0.154 |
| Lipids-genitalia | -0.123 | 0.496 | -0.120 |
| Proteins-foot muscle | -0.155 | -0.168 | 0.320 |
| Proteins-hepatopancreas | -0.112 | -0.123 | 0.344 |
| Proteins-genitalia | -0.086 | -0.208 | 0.273 |
| LDH-foot muscle | 0.409 | 0.274 | -0.263 |
| LDH-hepatopancreas | 0.408 | 0.285 | -0.232 |
| LDH-genitalia | 0.402 | 0.199 | -0.379 |
| Period | Active autumn | Active spring | Aestivation |
| (B) *Minimum squared Mahalanobis distances* | | | |
| Active spring | 166.698 | | |
| Aestivation | 225.036 | 379.943 | |
| Hibernation | 60.628 | 76.005 | 394.744 |

between are placed autumn and spring months of activity. The above suggest that the measured biochemical variables have a coordinated behaviour over time and are sufficient to distinguish the four biologically different periods.

### 3.2. Biochemical and water content changes

There was a significant effect of period on all biochemical variables (MANOVA: Wilks' Lambda = 0.012, $F_{12,\ 453} = 165.726$, $P < 0.0001$) and on water content (see ANOVA results, Table 3). Additionally, there was a significant effect of tissue type on all biochemical variables (MANOVA: Wilks' Lambda = 0.167, $F_{8,\ 342} = 61.745$, $P < 0.001$) but not on water content (Table 1). MANOVA indicated interaction of period × tissue type on all biochemical variables (Wilks' Lambda = 0.646, $F_{24,\ 598} = 3.318$, $P < 0.001$), and such interactions were supported for lipids, proteins and LDH in the ANOVA (Table 3). Although we found significant differences between biochemical and water content between tissue types, contents of particular biochemicals or water were generally highly correlated ($r > 0.9$, $P < 0.001$) across tissue types. Only in the cases of correlation of protein content in genitalia with that of the hepatopancreas ($r = 0.597$) and foot ($r = 0.638$) was the level of association more modest.

Fig. 2 shows the changes of glycogen, lipid, protein and water content, and of LDH activity for each tissue type, which occur between the life cycle periods. The pattern of monthly changes (not shown) of biochemical and water content, albeit more complicated, corresponded to that general pattern of seasonal changes described below (Fig. 2). From these results we can support that aestivation was characterized by a rapid decline of glycogen, lipid, protein and water content and a substantial rise of LDH activity in all tissues. Protein content of genitalia tissue exhibited a more modest decline. During the active autumn period there was a rise of glycogen, protein and water content and a decline of lipid content and LDH activity. These changes were more profound for protein content of foot tissue, and for water content of genitalia and hepatopancreas tissues. During hibernation glycogen, lipids, proteins and water content continued to rise whereas LDH continued to fall. Changes were more intense for lipid and water content of foot tissue. Finally, during the active spring period glycogen and lipid content reached their peak, whereas proteins and water content (except water of the hepatopancreas tissue) exhibited a moderate decline. During that period the increase of LDH activity began. Even though these patterns of change among periods were also affected by the type of tissue (Table 3), we can observe a more or less coordinated response of all tissue types to period. Therefore, we can support that although the effects of period and tissue type were not simply additive, period had a substantial effect on biochemical and water content. The magnitude of this effect may depend on the tissue type. Post-hoc comparisons (Bonferroni test) indicated the significant differences found in biochemical content between tissues across periods. Differences were found between genitalia and the other two tissues (i) in glycogen content for all periods except aestivation, (ii) in lipid content during hibernation, (iii) in protein content during hibernation and active spring, and (iv) for LDH activity in hepatopancreas during aestivation. Further, in all tissues: (i) LDH activity was significantly higher during aestivation, (ii) lipid content was higher during the active spring period, (iii) protein content was higher during hibernation, and (iv) water content was lower during aestivation.

With individual snails taking several years to reach maturity and presumably subsequently contributing to recruitment over several years, there is clearly a complex age structure in *C. helenae* populations. However, it seems that there was not considerable influence on the results of the samples comprising adults of different ages. The small error found in monthly or seasonal estimates (not shown for simplicity) suggests that there was little effect of these differences in adult ages.

### 3.3. Correlations between variables

We found significant correlations in monthly values for the following variables: glycogen and water content ($r^2 = 0.572$, $P < 0.003$), proteins and LDH ($r^2 = 0.742$, $P < 0.002$), proteins and water content ($r^2 = 0.487$, $P < 0.007$), and LDH with water content ($r^2 = 0.581$, $P < 0.002$).

There was a high correlation among climatic variables ($r > 0.9$ at $P < 0.001$). PCA resulted in a single component variable that accounted for 95% of total variance. This climatic component was in turn strongly correlated with LDH ($r^2 = 0.846$, $P < 0.0001$), proteins ($r^2 = 0.818$, $P < 0.0001$), and modestly correlated with water content ($r^2 = 0.501$, $P = 0.006$). Also, it was strongly correlated ($r^2 = 0.728$, $P < 0.0003$) with the first component variable (61.9% of total variance) from the PCA of mean monthly values of the measured variables.

Finally, we did not find any evidence for time lags in correlations (at 95% confidence interval) between the climatic component and the measured variables. Significant cross-correlations were, however, identified between glycogen

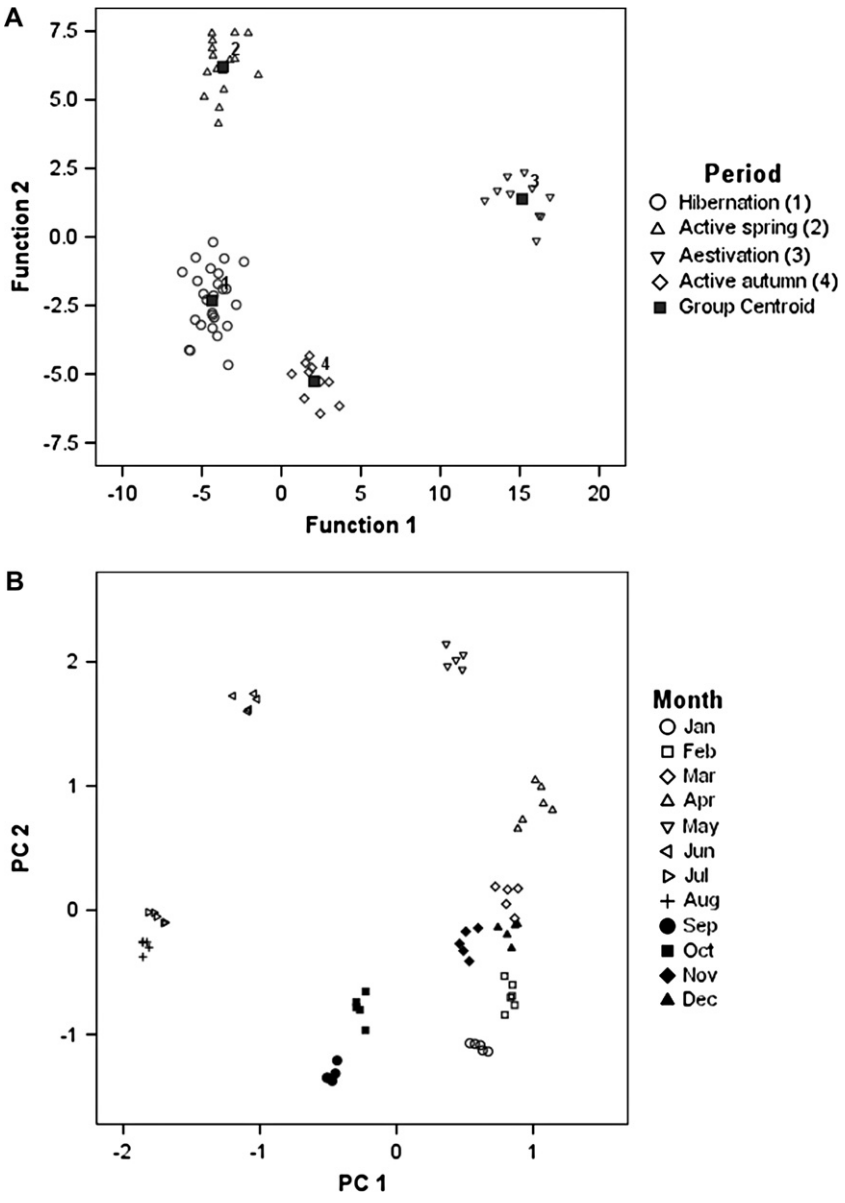


**Fig. 1 – (A) Discriminant function plot of periods, and (B) principal component analysis ordination plot of months. Each point represents an individual snail.**

and lipids (time lag: +1, r ± SE: 0.911 ± 0.316), between glycogen and LDH (time lag: +3, r ± SE: 0.826 ± 0.354), and between lipids and LDH (time lag: +2, r ± SE: 0.826 ± 0.333) These cross-correlations imply, for example, that glycogen may ''predict'' lipid content in tissues 1 month later, and LDH activity 3 months later. However, we should be cautious with such interpretations.

## 4. Discussion

The major outcome of our study was the unambiguous discrimination of the four life-cycle periods. Most remarkable was the clear distinction of the aestivation period, with hibernation, the other dormancy period, showing similarity with the two active periods and not with aestivation. These results indicate that the most dramatic physiological changes take place during aestivation. The observed rapid depletion of energy reserves and the severe decrease in tissue water content during aestivation may constitute a significant physiological stress and a threat to survival for *C. helenae*, not unlike that previously highlighted by Storey (2002) for other land snails. The important question, therefore, is 'are these physiological changes adaptations to a predictable summer dormancy requirement, or simply responses to prevailing stressful conditions?' Our results on biochemical and water content and enzyme activity indicate that physiological changes are initiated prior to aestivation and thus are at least partly adaptive.

We found lipids and glycogen at their highest concentrations at the start of aestivation, and were depleted during

**Table 2 – Results of the principal component analysis (PCA) on the biochemical variables: component matrix (eigenvectors), showing the contribution of each variable to the first three components**

| | Component | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Glycogen-foot muscle | 0.780 | 0.432 | 0.394 |
| Glycogen-hepatopancreas | 0.734 | 0.475 | 0.462 |
| Glycogen-genitalia | 0.779 | 0.427 | 0.442 |
| Lipids-foot muscle | 0.310 | 0.861 | −0.337 |
| Lipids-hepatopancreas | 0.118 | 0.841 | −0.449 |
| Lipids-genitalia | 0.096 | 0.913 | −0.243 |
| Proteins-foot muscle | 0.850 | −0.344 | −0.033 |
| Proteins-hepatopancreas | 0.813 | −0.407 | 0.096 |
| Proteins-genitalia | 0.578 | −0.580 | −0.362 |
| LDH-foot muscle | −0.932 | 0.209 | 0.226 |
| LDH-hepatopancreas | −0.925 | 0.198 | 0.251 |
| LDH-genitalia | −0.948 | 0.143 | 0.221 |

**Table 3 – Results of analysis of variance (ANOVA) for life cycle 'period' (hibernation, active spring, aestivation, active autumn), 'tissue type' (genitalia, foot muscle, hepatopancreas), and their interactions, on the glycogen, lipid, protein and water content, and LDH activity of *Codringtonia helena* (NS, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$)**

| Effect | Dependent variable | d.f. | *F*-ratio | *P*-value |
|---|---|---|---|---|
| Period | Glycogen | 3, 174 | 49.645 | *** |
| | Lipids | 3, 174 | 180.687 | *** |
| | Proteins | 3, 174 | 77.329 | *** |
| | LDH | 3, 174 | 576.232 | *** |
| | Water | 3, 62 | 15.611 | *** |
| Tissue | Glycogen | 2, 174 | 56.378 | *** |
| | Lipids | 2, 174 | 41.219 | *** |
| | Proteins | 2, 174 | 35.038 | *** |
| | LDH | 2, 174 | 46.213 | *** |
| | Water | 2, 62 | 0.542 | NS |
| Period × tissue interaction | Glycogen | 6, 174 | 0.624 | NS |
| | Lipids | 6, 174 | 2.157 | * |
| | Proteins | 6, 174 | 2.714 | * |
| | LDH | 6, 174 | 3.243 | ** |
| | Water | 6, 62 | 0.365 | NS |

the aestivatory dormancy. In contrast, the decline in protein content began earlier in the season and was possibly associated primarily with oviposition activity, and secondarily with the decline of tissue water potential and the increased demand for urea synthesis. Catabolism of carbohydrates seems to be of major importance for aestivating land snails (Livingstone and de Zwaan, 1983), and indeed, our results resemble those for lipids and polysaccharides observed by Giokas et al. (2005) in another rock-dwelling land snail in Greece, *Albinaria caerulea* Deshayes, 1835. In a 7-month aestivatory period in *Oreohelix strigosa* (Gould, 1846) and *O. subrudis* (Reeve, 1854), Rees and Hand (1993) found polysaccharides to be the primary metabolic fuel for the first 2–4 months, and only when these sugars were depleted did protein catabolism begin (a low rate of lipid catabolism was maintained throughout).

Survival during aestivation is aided by any physiological mechanism that prevents exhaustion of fuel reserves before the return of environmental conditions favourable for general activity. Metabolic rate in land snails during aestivation is low, usually 10–30% of the metabolic rate in active individuals (Herreid, 1977; Pedler et al., 1996), and obviously the greater the reduction in metabolic rate, the longer the time that a fixed reserve of fuels can sustain basal metabolism. Part of the reduction in metabolic rate is the result of cessation in digestion and locomotor activity, and part is due to reduced rates of breathing and heart beat, as well as to apnoeic breathing and its consequent effects on pH and oxygen utilization in oxyconforming species (Storey, 2002). However, a significant saving in fuels results from a coordinated reduction in the rates of energy turnover in tissues. Decreased rate of fuel catabolism, ion channel arrest, and reduced rates of protein synthesis are among the factors that contribute to intrinsic metabolic rate depression (Churchill and Storey, 1989; Storey and Storey, 1990; Rees and Hand, 1991; Guppy et al., 1994). In *C. helenae* we have some indications that the reduced rate of protein synthesis is a mechanism for metabolic depression. However, the situation in *C. helanae* is confounded since aestivation is not infrequently disrupted as individuals become active over short periods when climatic conditions are temporarily favourable.

This reduced metabolism in aestivating land snails is generally regarded as aerobic, with minimal employment of anaerobic pathways (Rees and Hand, 1990; Brooks and Storey, 1997; Michaelidis, 2002). Glycolysis is down-regulated and internal oxygen levels are adjusted, albeit at a low level, by precise control of gas exchange. Because aestivating land snails are aerobic, regulation of the terminal end of glycolysis is different from that found in anoxic animals (Brooks and Storey, 1997; Withers et al., 1997; Michaelidis, 2002). Usually, aestivating land snails do not show increased LDH activity since they completely oxidize carbohydrate (Churchill and Storey, 1989; Brooks and Storey, 1997), contrary to several vertebrates where LDH has a crucial role in metabolism when oxygen levels are low (Somero, 1973; Bennett, 1974; Wolfe et al., 1988). However, in our study of *C. heleanae*, we have found elevated LDH activity during aestivation.

In *C. helenae* the rise of LDH starts from the end of hibernation period, is more abrupt during aestivation, and reaches a peak during the last month of aestivation (August). This pattern contrasts with that reported for most aestivating land snails (see Brooks and Storey, 1997), but again is similar, yet not identical, with what we have observed in *A. caerulea* (Giokas et al., 2005). In that species the rapid accumulation of LDH does not occur before the first weeks of aestivation. Interestingly, the range of variation in LDH levels is actually the same in both species. There is evidence that, at certain times during aestivation, energy production in land snails may be based on anaerobic processes (Wieser, 1978; Wieser and Wright, 1978, 1979; Michaelidis et al., 1999) and this may apply to *C. helenae*. Anaerobic metabolism seems to occur in dormant pulmonates because the rate of oxygen consumption falls below measurable limits for hours or even days (Schmidt-Nielsen et al., 1971). Terrestrial snails may find themselves in oxygen-free conditions, e.g. when they burrow

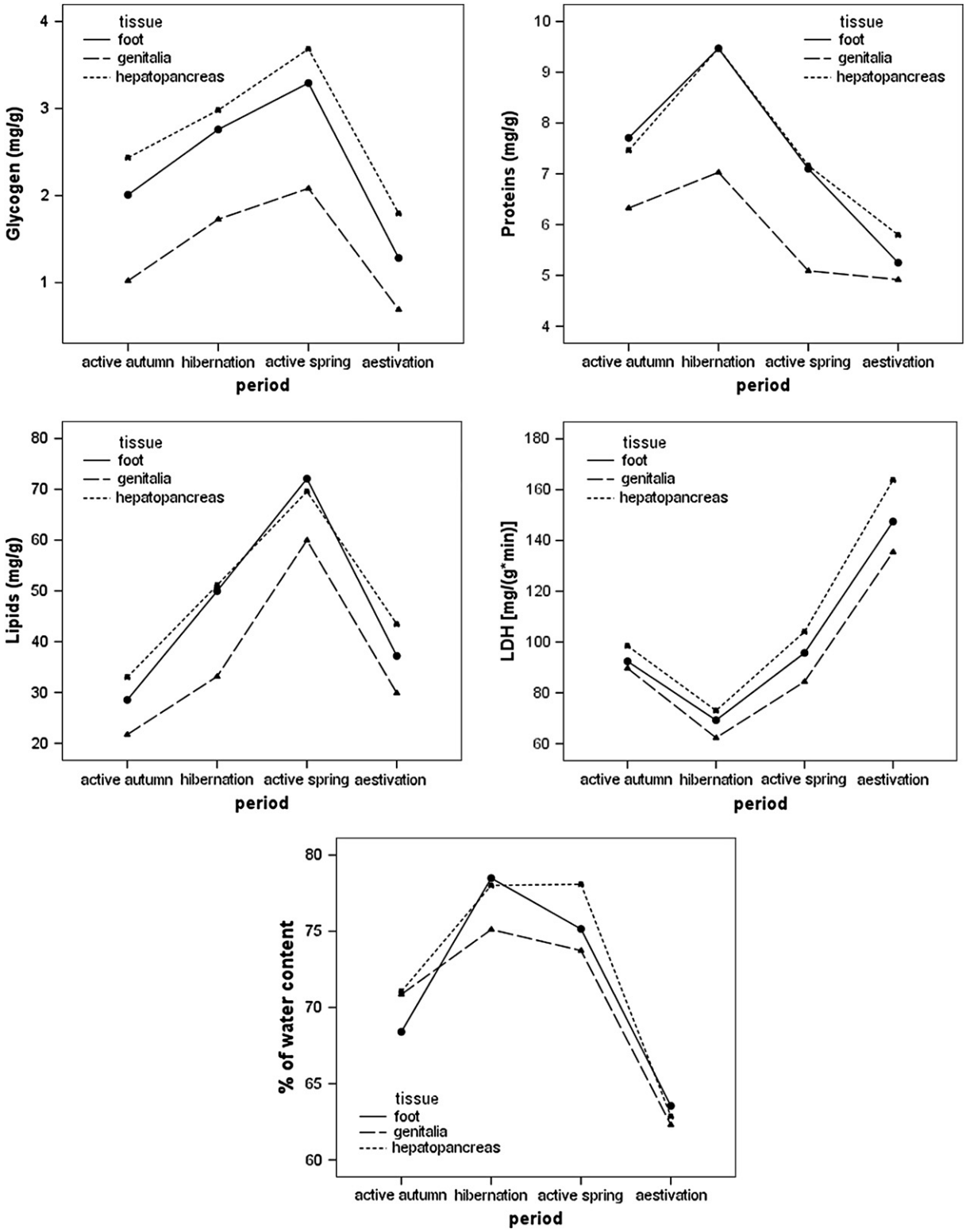


**Fig. 2 – Changes of biochemical content (glycogen, lipids, and proteins), LDH activity, and % of water content between periods. Values are given as means.**

deep in the ground (Von Brand, 1944). Furthermore, Wieser and Wright (1978) have shown that land snails process a high glycolytic potential that may be used for anaerobic energy production even when the animal's environment appears to provide an adequate supply of oxygen. Further, long-term acclimation to elevated $CO_2$ levels can induce an increase in the activity of LDH with a concomitant accumulation of D-lactate in tissues (Michaelidis et al., 2007). This indicates that long-term acclimation to elevated ambient $CO_2$ levels could reduce the aerobic capacity of land snails and trigger

expression of anaerobic pathways. However, this idea of anaerobic metabolism in land snails is still controversial (Michaelidis et al., 1999). The correlation we found between the seasonal activity of LDH in *C. helenae* and climatic factors such as temperature, has also been observed in studies of *Helix pomatia* Linnaeus, 1758 (Wieser and Wright, 1979) and on *A. caerulea* (Giokas et al. (2005)), and may indicate compensatory acclimation of this energy-associated enzyme. More work is clearly needed to explain fully the rise of LDH during aestivation in *Codringtonia*. In any case, the increase in LDH implies that to aestivation is a stressful period demanding supplementary energy.

Aestivating snails often show adaptations that retard water loss during dormancy. Because water is lost during breathing and also across the skin/epithelium layer, snails are thought to normally enter aestivation with large reserves of body water that can be drawn upon to keep tissues hydrated (Storey, 2002). However, in our study we have measured the highest level of water content in April, 3 months before the start of aestivation, and moreover the rapid decline of water starts during the active spring period. Therefore, *C. heleanae* enters aestivation with relatively low water reserves that are rapidly depleted.

The above suggest that *C. helenae* cannot efficiently manage water budgeting during aestivation. Therefore, *C. helenae* possibly lacks trade-off mechanisms that may reduce water loss. Such mechanisms have been suggested for land snails during aestivation and include: apnoeic breathing patterns during breathing that minimize evaporative water loss (Barnhart, 1983; Barnhart and McMahon, 1987), elevation of the osmolarity of body fluids due to the establishment of high concentrations of solutes such as urea (Rees and Hand, 1993; Arad, 2001). Arad and Avivi (1998) and Arad (2001) have also suggested that during long-term aestivation, a new set point of water economy is established in association with metabolic depression. Yom-Tov (1971a,b) and Arad and Avivi (1998) suggested that snails regulate their water budget through metabolic pathways by oxidation of storage substrates that contribute extra metabolic water. Steinberger et al. (1982) have estimated that in *Sphincterochila prophetarum* Bourguignat, 1852 metabolic water contributes about 8% to the total water economy during aestivation, and partly compensates for water loss. The fact that *C. helenae* lacks efficient water regulation mechanisms might be explained by recalling that the species, although living in southern Greece, has a life cycle (oviposition during spring, main dormancy period during winter, short occasional aestivation period) similar in many ways to land snails of more northern regions. The life cycle of *C. helenae* might represent relictual adaptations to past environmental conditions (between 15,000 and 6000 years ago) when the herb-steppe of the late Pleistocene Mediterranean region was replaced during the early and mid-Holocene by subhumid forest, sometimes dominated by conifers, but more usually by broad-leaved deciduous trees (Roberts et al., 2001).This apparent disassociation between behavioural ecology and physiological responses, at least as far as water regulation is concerned, likely constitutes a major problem for the survival of *C. helenae* in southern Greece, especially in areas where the favourable vegetated habitats are further reduced by human activities. This poor adaptation to the Mediterranean environment may deteriorate further by predicted climate change due to global warming. Giokas et al. (2005) have found that in contrast to *C. helenae*, *Albinaria caerulea* is well adapted to arid conditions and exhibits a low rate of decline of water content during aestivation and insignificant differences in mortality between the active and aestivation periods. However, to obtain a better picture of the consequences of the apparent mismatch between life cycle and prevailing environmental conditions, it would be useful to have estimates of mortality for *Codringtonia* species.

The disassociation between behavioural ecology and physiological responses of *C. helenae* is also evident for the hibernation period. In *Codringtonia* hibernation is considered a true dormancy period since in the field, all animals remain inactive throughout that period deep in rock-crevices, forming 5–6 layers of membranous semi-transparent epiphragms over the shell aperture, and dormancy is not interrupted when climatic conditions are favourable (Hadjicharalambous, 1996, and personal observations). On the contrary, aestivation is considered as an ''occasional'' dormancy because, when climatic conditions are favourable (low temperatures, high humidity and rains), a part of the population becomes temporarily active. However, our data for *C. helenae* support that the physiological responses during hibernation constitute a continuum linking the active autumn and active spring periods, since fuel reserves and water content do not decline drastically during hibernation and LDH remains at low levels from the start of the active autumn period until the end of the active spring period (see Figs. 1 and 2). This pattern indicates that drastic physiological changes are not associated with hibernating behaviour, and that hibernation is not a stressful period (or at least *Codringtonia* copes efficiently with environmental conditions during hibernation both in behavioural and physiological terms).

According to Cook (2001) there are two elements in the control of the onset and termination of aestivation. First, there is a pre-programmed element that is controlled by a circannual rhythm entrained by day length and which involves physiological preparations and an increased tendency to adopt habitual summer resting sites. Second, this preparation may be overridden by events and animals may be forced into a dormant state by prolonged dehydration, high temperatures etc. However, we observed that *C. helenae* enters in aestivation late relative to the climatic conditions of summer, and terminates aestivation before the actual start of the autumn rainy season. Additionally, even though it would be advantageous to not become active during temporary periods of low temperature or occasional heavy rains in the summer because of a short-term fall in temperature, or occasional heavy rains, *C. helenae* exhibits occasional or disrupted aestivation behaviour. Cook (2001), summarising relevant information, states that aestivation in shelled terrestrial gastropods may only have a very weak endogenous component, being largely controlled both in its onset and its termination by the prevailing climatic environment. However, we found strong correlation of climatic conditions only with LDH and proteins (and marginally with water content). Yet, interestingly, the biochemical variables, taken as a whole, are sufficient to distinguish the period of aestivation (Fig. 1). Therefore, perhaps the start of aestivation is primarily controlled by an endogenous

component and changes in the biochemical composition are a by-product of this component and not a direct response to climatic conditions. On the other hand, the biochemical mechanisms used for metabolic suppression in aestivation need to be effective, but easily reversible, to allow a rapid return to normal metabolism during arousal (Storey, 2002). In the present study we have found that glycogen, lipids, and proteins tend to accumulate rapidly after arousal.

## 5. Conclusion

In conclusion, our study showed that the physiological responses of *C. helenae* are effective during hibernation, but only partly compensate the effect of adverse environmental conditions during aestivation, since the aestivating behaviour of that species is occasional and time limited. Perhaps, the behavioural ecology of *C. helenae* is in large part relictual and has been shaped in past more humid and cold environmental conditions (Roberts et al., 2001). This constitutes an important extinction threat if we consider the current trends of climatic conditions and the human impact on vegetation cover in the preferred habitats of that species. However, it is difficult to make a conclusive argument about whether these responses are adaptive since we lack estimations of parameters directly related to fitness (e.g., growth, fecundity, survival, etc.). Further comparative studies on the ecology and physiology of additional *Codringtonia* populations (and indeed other land snails) would aid our understanding of natural selection, especially if we consider physiological changes as trade-offs with consequences for fitness. Only then we will be able to clarify the impact of climatic changes on the Mediterranean biodiversity hot spot.

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