

ECOLOGICAL AND PHYSIOLOGICAL ADAPTATIONS OF THE LAND SNAIL *ALBINARIA CAERULEA* (PULMONATA: CLAUSILIIDAE)

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ABSTRACT

We examined the seasonal patterns of physiology and biochemical composition in the rock-dwelling clausiliid land snail *Albinaria caerulea* in relation to its ecology, morphology and climatic data. We found that *A. caerulea* displayed significant seasonal changes in biochemical composition and enzyme activity, especially during aestivation. Sugars were catabolized early in aestivation, and proteins and lipids seem to be the primary metabolic substrates after sugar reserves were depleted. Atypical for aestivating land snails was the accumulation of lactate and LDH during the first weeks of aestivation, possibly suggesting that anaerobic pathways can provide additional energy. Moreover, mortality was not significantly higher during aestivation, fresh-mass differences between the aestivation and active period were not substantial and the rate of fresh-mass decline during aestivation was low. In addition, *A. caerulea* shows a series of morphological (thick white shell, strengthened and reflected apertural lips, thick epiphragm, internal lamellae, door-like clausilium) and behavioural (high aggregation) characteristics that support survival during adverse climatic conditions by preventing desiccation. We did not find a correlation of biochemical variables (except LDH) with climatic conditions. Yet, the biochemical variables, taken as a whole, are sufficient to distinguish the periods of aestivation and activity. The above suggest that the start of aestivation is primarily controlled by an endogenous component, and perhaps changes in the biochemical composition are a by-product of this component and not a direct response to climatic conditions. These results, in conjunction with the non-elevated mortality during aestivation, indicate that in this *Albinaria* population morphological, behavioural and physiological adaptations to the dry conditions of the climatically unpredictable Mediterranean-type ecosystems are in effect, and seem to ensure sufficient water and fuel reserves.

INTRODUCTION

Land snails in seasonally arid or semi-arid regions experience annual cycles of activity and aestivation, and therefore, should be adapted with a range of behavioural and physiological mechanisms that will ensure their survival under adverse environmental conditions. Seasonal variations in land snail physiology and biochemical composition have been linked to annual cycles of photoperiod, temperature, humidity, water availability and reproduction (Machin, 1975; Riddle, 1983; Cook, 2001; Storey, 2002). Seasonal physiological data for land snails may be useful in understanding species-specific habitat requirements and in predicting their response to environmental changes. Of special interest are responses and adaptations of land snails to the climatically unpredictable, but usually semi-arid Mediterranean-type ecosystems (Blondel & Aronson, 1999).

There is excellent ongoing experimental research on the metabolic pathways in land snails, especially during aestivation or under anoxia (for a review see Storey, 2002). However, there are relatively few informative, long-term field studies on aspects of the physiological ecology of land snails (Wieser & Wright, 1979; Riddle, 1983; Arad, 1990, 1993; Rees & Hand, 1993; Withers, Pedler & Guppy, 1997; Arad & Avivi, 1998; Arad, Goldenberg & Heller, 1992, 1998). Likewise, very few data are available on the biology and ecophysiology of the widespread pulmonate family Clausiliidae, especially in semi-arid or arid Mediterranean-type ecosystems (Warburg, 1972; Piechocki,

1982; Baur, 1990; Baur & Baur, 1991; 1992; Heller & Dolev, 1994; Arad, Goldenberg & Heller, 1995).

Albinaria Vest, 1867 is the most prominent and speciose clausiliid genus. This rock-dwelling genus is distributed around the northeastern coasts of the Mediterranean, exhibiting high morphological and molecular differentiation, especially in southern Greece (Douris *et al.*, 1995; Douris, *et al.*, 1998a; Douris, *et al.*, 1998b; Schilthuizen, Gittenberger & Gulyaev, 1995; Giokas, 2000; Van Moorsel, Dijkstra & Gittenberger, 2000). As a consequence more than 90 (Nordsieck, 1999), sometimes dubious (Douris *et al.*, 1995, 1998a,b; Van Moorsel *et al.*, 2000), usually narrowly endemic, species have been described and *Albinaria* species constitute about 15% of the total Greek land snail fauna. *Albinaria* is considered to be a model organism and as a result there are many studies on its biogeography, phylogeny and evolutionary genetics (for reviews see Giokas, 2000; Van Moorsel, 2001). However, there are few studies on ecological differentiation and life history in *Albinaria* (Gittenberger, 1991; Giokas & Mylonas, 2002) and ecophysiological studies are lacking. Nevertheless, research on the physiological ecology of *Albinaria* can be very informative for inferring evolutionary processes and physiological adaptations in the semi-arid eastern Mediterranean region, because interestingly, the high number of *Albinaria* species in these relatively hostile environments does not seem to be associated with a matching degree of ecological differentiation (Gittenberger, 1991; Welter-Schultes, 2000; Giokas & Mylonas, 2002).

This is the first study on the seasonal physiological responses of *Albinaria*. We examined, over 1 year, sequential monthly samples of adult specimens of *Albinaria caerulea* (Deshayes, 1835),

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a representative *Albinaria* species, for changes in fresh mass, biochemical tissue composition (sugars, lipids, proteins and lactate), catabolism of energy reserves, and activity of lactate dehydrogenase (LDH), an enzyme associated with anaerobic pathways, catalysing the interconversion of pyruvate and lactate. We analysed these results and examined their association with climatic and life-history records (mortality, aestivation behaviour) and morphological features. The aim of this study was to reveal potential physiological, behavioural and morphological adaptations and seasonal patterns that may explain the prevalence of *Albinaria* in the semi-arid environments of southern Greece. Furthermore, we hope that this work can serve as a basis for future comparative studies on the ecophysiology of clausiliids.

MATERIAL AND METHODS

The study population

Albinaria caerulea lives, like most *Albinaria* species, on limestone substrates in semi-arid habitats, and aestivates on limestone rock-surfaces covered by lichens, algae and mosses, or around rock crevices and occasionally on shrubs, while forming a thick epiphragm (Giokas & Mylonas, 2002). *Albinaria caerulea* has a moderately sized, smooth, white elongate shell, up to 16 mm in height. It typically occurs in large numbers, especially in the study population, despite mortality resulting from desiccation during aestivation or weakening after oviposition (Giokas & Mylonas, 2002), or from occasional heavy predation during aestivation by beetle larvae, rodents or birds (Schilthuizen, Kemperman & Gittenberger, 1994). In the study population the active season starts at the onset of autumn rains, around mid-October, and ends with the beginning of the dry season, in late April (Giokas & Mylonas, 2002). Copulation starts immediately after activation and eggs are laid approximately 3 weeks later (Giokas & Mylonas, 2002). In nature, the development from a juvenile to a fully-grown snail takes two, or occasionally three, wet seasons and the estimated life span of *A. caerulea* is about 7 years (Giokas, 1996). During the last dry season before sexual maturation, the genital organs of the sub-adult snail increase in size and become mature (Giokas, 1996; Schilthuizen, 1994).

The study area

The study area was the Acropolis Hill adjacent to the archaeological site of Vravrona (about 35 km east from Athens, 37°56'N, 24°01'E, 153 m above sea level). This area belongs to the intense thermo-Mediterranean bioclimatic zone, with semi-arid floor (between 125 and 150 biologically arid days), and warm sub-floor (mean minimum temperature of the coldest month of the year above 7°C) (Mavrommatis, 1978). Other climatic data are shown in Table 1. There are large blocks of limestone, forming physical corridors suitable for these rock-dwelling snails, with, more rarely, smaller blocks scattered in between. The

terrain lacks large vegetation other than tough shrubs ('phrygana' and 'maquis') (for definitions see Blondel & Aronson, 1999).

Sampling

Random samples were taken every month from April 1999 until March 2000, always on dry days during the first week of each month, at approximately 09.00 h. For the detailed sampling procedure (quadrat counts method) see Giokas & Mylonas (2002). In each monthly sample, we recorded all the adult and juvenile specimens as well as their condition (alive, dead, aestivating and active).

Estimations of body mass and biochemical composition

Individuals were taken alive to the laboratory within 3 h of each sampling. Specimens were immediately weighed (after removing the shell), and tissues were frozen in liquid nitrogen so that the metabolites would be preserved. Foot muscle tissue was collected from each animal and stored at -80°C .

For lactate determination, muscle tissue (approximately 150 mg) from each snail was homogenised (1:3 w/v) with 10% ice-cold perchloric acid in a cold pestle on ice. The homogenate was centrifuged at 5,000 g for 10 min at 4°C. The supernatant was neutralized with 0.5 M Tris/0.5 M KOH and centrifuged at 10,000 g for 10 min at 4°C. The pellet was discarded and the supernatant used for the determination of total lactate concentration according to the method described by Hohorst (1965).

Total lipids were extracted by homogenizing the muscle tissue (30–40 mg) with 1.5 ml of a mixture containing two parts chloroform to one part absolute methanol. The homogenate was centrifuged at 3,000 g for 10 min at 4°C. The pellet was discarded and the supernatant was used for the determination of total lipid concentration, using an appropriate kit (Chromatest) according to the method described by Alexis & Papaparaskeva-Papoutsoglou (1986). A mixture of olive and corn oil (2:1 v/v) was used as the standard.

Total sugars were determined according to Dubois, Gilles, Hamilton, Rebers & Smith (1956). Tissue (150 mg) was homogenized in distilled water (1/10 w/v) and boiled at 100°C for 30 min. From each sample 20 µl were diluted (1:500 in water) and incubated with 1 ml phenol (5% w/v) and 5 ml of 95% H₂SO₄. After incubation at room temperature for 10 min, further incubation was performed at 30°C for 40 min and the absorbance was read at 490 nm.

Total proteins were determined by the Biuret method. The centrifuged pellet was dissolved with 0.5 ml of 0.1N NaOH and incubated at 37°C for 30 min with sporadic vortexing. Fifty ml of each sample was diluted with 950 µl water and then 4 ml of diluted Biuret reagent were added. The mixture was incubated for 30 min at room temperature and the absorbance read at

Table 1. Climatic data for the study site (source: National Observatory of Athens).

Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug.	Sept	Oct	Nov	Dec	Year
Mean ambient temperature (°C)	9.3	9.8	11.7	15.5	20.2	24.6	27.0	26.6	23.3	18.3	14.5	11.1	17.6
Mean maximum ambient temperature (°C)	12.9	13.6	16.0	20.3	25.3	29.8	32.6	32.3	28.9	23.1	18.6	14.7	22.3
Mean minimum ambient temperature (°C)	6.5	6.9	8.4	11.6	15.4	20.1	22.5	22.3	19.2	14.9	11.4	8.3	14.0
Mean precipitation (mm)	44.6	48.3	42.6	28.2	17.2	9.7	4.2	4.6	11.9	47.7	50.6	66.6	376.1
Average number of raining days	13	13	11	10	7	5	2	2	3	9	11	13	99
Average relative humidity (%)	72	71	68	62	58	52	48	49	56	66	73	73	62
Mean absolute humidity (mm Hg)	6.4	6.5	7.0	7.9	9.7	11.4	12.4	12.4	11.7	10.3	9.0	7.3	9.3
Mean sunshine duration (hours)	138	145	187	239	303	341	374	356	283	218	164	136	2884

550 nm. Bovine serum albumin was used (0.5–10 mg) as the standard.

Activity of LDH was measured according to Ward, Castro & Faibrain (1969). The tissue, 0.1–0.15 g, was homogenized (in the ratio 1:10) with a special solution (0.1 M Tris-HCl pH 7.5, 1 mM EDTA). The homogenate was centrifuged at 12,000 rpm for 1 min at 4°C. 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 began with the addition of 50 µl of the homogenate tissue. Subsequently, absorption reductions (at 340 nm) were measured per min. The activity of LDH was calculated using the following formula: $\Delta OD \times 3.05 \times \text{dilution}$ (the dilution in *Albinaria*'s tissue was $\frac{1}{2}$).

Data analysis

Spatial distribution in the field was estimated using: (a) Green's index of dispersion:

$$GI = \frac{s^2/\bar{x} - 1}{\sum(X) - 1}$$

(\bar{x} = mean sample density, s^2 = sample variance, and $\sum(X)$ = sum of the quadrat counts), and (b) Taylor's Power Law ($\sigma^2 = a\mu^b$) (where σ^2 = variance, μ = arithmetic mean of population, a and b are population parameters) (Elliot, 1971; Ludwig & Reynolds, 1988; Hayek & Buzas, 1997). Parameter b , in Taylor's Power Law, can vary from zero to infinity. When $b > 1$ the distribution is contagious, when $b = 1$ the distribution is random and when $b < 1$ the distribution is uniform. Negative values of Green's index indicate a uniform pattern and positive values indicate a clumped pattern. For each sample mortality was estimated using the ratio: number of dead specimens/(number of alive + dead specimens).

Based on field observations we defined two periods: the aestivation period (samples from May to September), and the active period (samples from October to April). We examined the effect of collection date (month and period) on the biochemical measures (lipids, lactate, proteins, sugars and LDH) and fresh mass. We analysed all variables, except fresh mass, simultaneously with multivariate analyses of variance (MANOVA), followed by univariate analyses (ANOVA). Moreover, we used regression analysis to examine the rate of decline or increase of biochemical variables and fresh mass during aestivation. In addition, we examined possible autocorrelation, and partial correlation of each mean monthly measure at other time lags using time series analysis. In order to identify relations between pairs of mean monthly measures and possible time-delayed effects we used correlation and covariance analysis and subsequently we examined, using time series, cross-correlations of these estimates. Furthermore, we used stepwise discriminant analysis (SDA) and logistic regression analysis (LRA) in order to determine which of the measured variables account most for the differences between the two periods. Finally, we analysed the structure of interrelationships among the measured variables using principal components analysis (PCA). For the perceptual mapping of months we used the factor scores of PCA for each month, and we also applied multidimensional scaling (MDS) for all the mean monthly values of each variable. We then examined the correlation (and partial correlation) of the variables measured with the series of climatic data for the sampling site shown in Table 1. All the statistics used are described in Zar (1984) and Hair *et al.* (1998).

RESULTS

Behaviour and mortality

In the middle of April, 75% of the adult sample ($n = 177$) had formed an epiphragm, while at the end of April, 95% of them were fully aestivating. The onset of the aestivation period was independent of the end of rainfall. Activation and copulation occurred only after the first autumn rains (in October), and never after occasional rains during summer or in September. In mid-September we did not observe any specimens with fragmented epiphragms, but 2 weeks later, at the beginning of October, in 81% of the adults ($n = 80$) the epiphragm was absent or extensively fragmented, and by mid October, all specimens lacked any trace of an epiphragm. Copulation was actually immediate and synchronous, and it was the main activity of *Albinaria* after becoming active. Copulation occurred over a period of a week. Oviposition started about 20 days after copulation, and stopped 10 days later. Eggs were laid in crevices or under stones, in clutches of about 5–8 eggs. Hatching occurred approximately 2–3 weeks after oviposition.

Spatial distribution was contagious in all monthly samples, according to the parameters a and b of Taylor's Power Law ($a = 0.5$, $b = 2.4 \pm \text{SE } 0.25$), and Green's Index (mean $GI = 0.17$, min $GI = 0.02$, max $GI = 0.64$). GI values were on average higher during aestivation (mean aestivation $GI = 0.36$).

Mean estimated mortality was generally low (10.5%). Mortality was on average slightly higher during aestivation (11.7%) than during the active period (10.47%), but there was no significant difference found between the two periods (ANOVA: $F_{1,13} = 0.08$, $P = 0.78$). Higher mortality of adult specimens was estimated for the two months after hatching (on average 17%).

Changes of biochemical composition

Figure 1 shows the monthly changes in tissue biochemical content (lipids, sugars, lactate and proteins), and LDH activity. Lipids were high at the beginning of the aestivation period but subsequently fell until its end (in September). There was a linear decline of lipids during aestivation (lipid concentration = $141.92 - 13.0683 \times \text{duration of aestivation}$; $F_{1,29} = 160.92$, $P = 0.0001$, $r^2 = 0.85$). Afterwards, there was an increase in lipid content until December, then a decrease, followed by an abrupt accumulation through to the end of the active period. Similarly, snails entered aestivation with a high sugar content, followed by an abrupt fall. Sugar levels remained low until the end of aestivation then showed a steady increase throughout the active period. Proteins revealed a more complex pattern. Protein content was constant during the first 3 months of aestivation and then dropped. Subsequently, at the beginning of the active period, proteins exhibited an abrupt increase and remained high for 4 months, and then decreased rapidly.

Lactate content showed an increase at the beginning of the aestivation period and a drop towards its end. Subsequently, lactate levels remained low throughout the active season. The activity of LDH increased gradually with the start of aestivation and decreased only after August (later than lactate). Then LDH remained, like lactate, at a very low level throughout the active period.

Significant autocorrelation and partial correlation (after partialing-out the influence of intervening points) was found only for LDH at time lag = 1 ($r = 0.703$, $\text{SE} = 0.256$, Box-Ljung = 7.548, $P = 0.006$). This implies that each value of LDH is highly correlated with the value of LDH at the previous time point (1 month earlier). We did not find any significant correlations among these variables (95% confidence level). However, we found a significant cross-correlation between

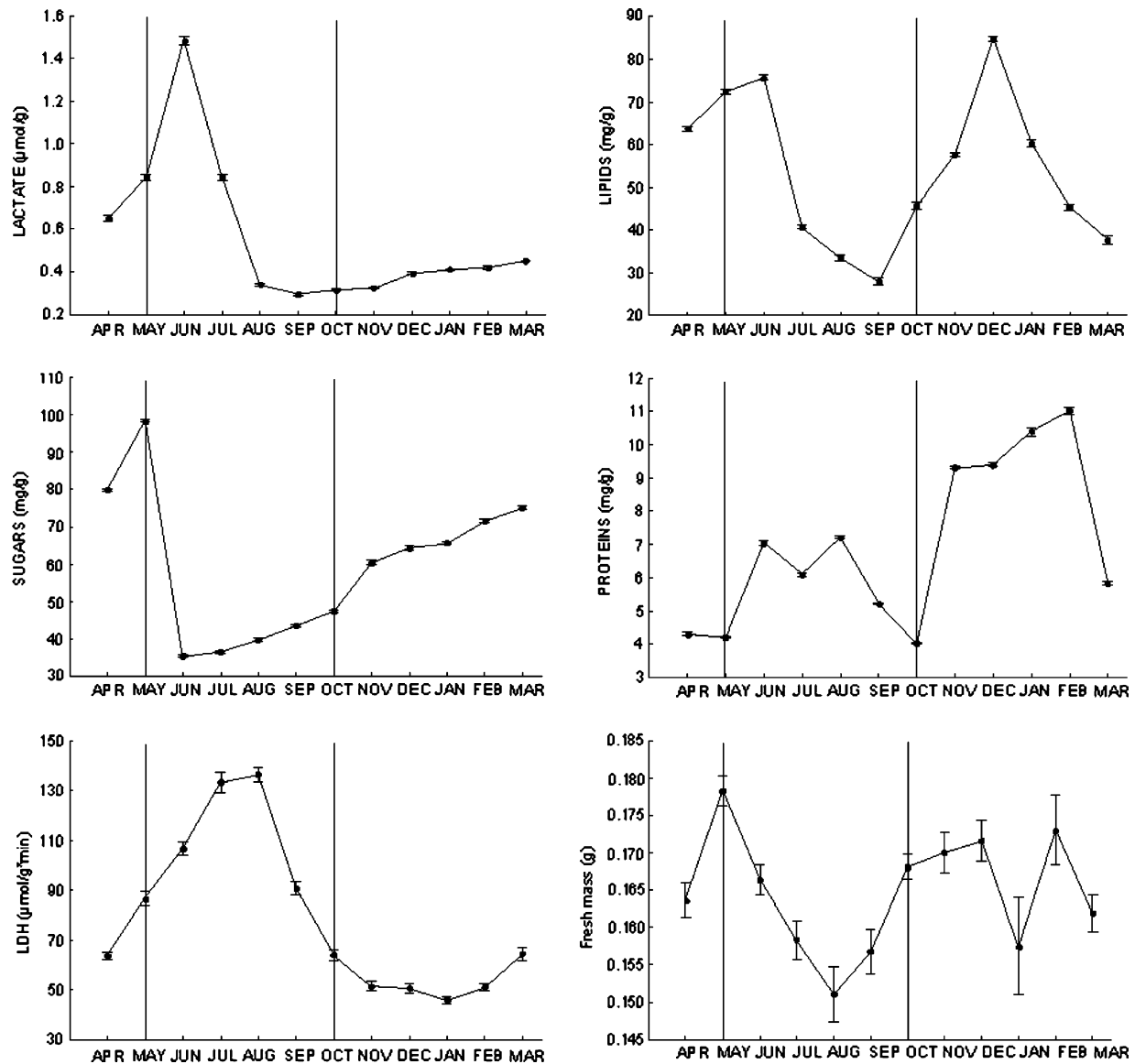


Figure 1. Monthly changes in muscle tissue biochemical content (lipids, sugars, lactates and proteins), LDH activity, and fresh mass. Values are given as mean \pm SE. Vertical lines delimit the aestivation period.

lactate and LDH at time-lags: +1 and +2, ($r \pm$ SE: 0.791 ± 0.302 and 0.766 ± 0.316 , respectively) implying that lactate content may ‘predict’ LDH activity 1 month and 2 months later or, in other words, high values of lactate content are positively correlated with high values of LDH 1 and 2 months later.

There was a significant effect of month on all the biochemical content variables, (MANOVA: Wilks’ Lambda < 0.001 , $F_{55,198} = 604.735$, $P < 0.0001$; and Table 2 ANOVA). These variables exhibited significant changes over time with relatively few statistically similar months (Table 2). In addition, there was a significant effect of period (active and aestivation) on all variables, except lipids (MANOVA: Wilks’ Lambda = 0.213, $F_{5,52} = 38.532$, $P < 0.001$; and Table 2 ANOVA). Sugars, proteins and lipids (not significantly) were, on average, higher during the active period. Lactate content and LDH activity were higher during aestivation. Similar outcomes resulted from an ANOVA when we standardized the above variables with the ratio: mean per month fresh mass/mean dry mass.

Discrimination of aestivation and active period

According to the SDA, LDH and lactate, in that order, were the two variables that accounted for most of the differences between the active and aestivating periods (95.9% of the cases were correctly classified). For LDH and lactate respectively: minimum squared mahalanobis distance between groups = 11.973 and 13.829, Exact $F = 170.311$ and 96.599 , $P < 0.0001$ and $P < 0.0001$. LRA, which is stricter, also revealed that LDH accounted for most of the differences between the two periods ($R = 0.7247$, d.f. = 1, $P < 0.00001$). Moreover, for lactate, according to LRA: $R = 0.389$, d.f. = 1, $P = 0.0002$.

The results of the PCA analysis are shown in Table 3. Components 1, 2 and 3 account for 90.4% of variation (Table 3A). LDH and sugars accounted for Component 1, lactate and lipids for Component 2, and proteins for Component 3 (Table 3B). The plot of the factor scores for Components 1 and 2 is shown in Figure 2. An identical picture resulted from MDS analysis; therefore the MDS plot is not presented. In PC1 there is a clear distinction between the aestivation months (except May) and the

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Table 2. ANOVA: effects of sampling month and period (aestivation and active) on biochemical composition and LDH activity.

Variable	Effect	d.f.	F
Lactate	Month	11, 62	945.92***
	Period	1, 72	30.24***
Lipids	Month	11, 62	632.48***
	Period	1, 72	0.79 ^{ns}
Sugars	Month	11, 62	2161.30***
	Period	1, 72	10.03**
Proteins	Month	11, 62	1445.99***
	Period	1, 72	13.11**
LDH	Month	11, 46	170.98***
	Period	1, 56	170.31***

ns = not significant,
^{*} $P \leq 0.05$,
^{**} $P \leq 0.01$,
^{***} $P \leq 0.0001$.

active winter months. In between are the autumn and spring months of activity. In PC2 there is a clear separation between the early and late months of aestivation. This suggests that the measured biochemical variables have a coordinated behaviour over time and are sufficient to distinguish biologically different periods.

Correlation with the climatic variables

Only LDH was highly correlated ($r \geq 0.9$, at $\alpha = 0.001$) with all the climatic factors examined. Among all the other variables, only sugars were marginally correlated ($r = 0.585$ – 0.615 , at $\alpha = 0.005$) with mean, maximum and minimum temperature, number of rainy days and absolute humidity. However, sugars were no longer significantly correlated with the climatic factors when we took into effect LDH. In addition, there was a significant correlation of the PC1 factor scores with all the climatic variables, possibly because LDH and sugars contribute more to PC1 (Table 3B).

Table 3. Principal component analysis of biochemical variables: A. Eigenvalues, percent of variance, and cumulative percentage. B. component matrix (eigenvectors).

A			
Component	Eigenvalues	% of variance	Cumulative %
1	2.023	40.468	40.468
2	1.486	29.719	70.187
3	1.007	20.148	90.335
4	0.300	5.991	96.326
5	0.184	3.674	100.000

B			
	Component		
	1	2	3
Lactate	-0.344	0.884	0.149
Lipids	0.522	0.777	0.175
Sugars	0.751	0.177	-0.527
Proteins	0.470	-0.218	0.822
LDH	-0.921	0.143	0.0325

Changes of dry and fresh mass

We measured dry mass (DM) in three samples only (March, July and November). Dry body mass did not differ significantly between these months (mean DM = 0.104 g, $F_{2,11} = 0.41$, $P = 0.675$). However, we found significant fresh-mass differences between the monthly samples ($F_{11,59} = 6.76$, $P < 0.0001$). It seems that *Albinaria* enter aestivation with large reserves of body water (Fig. 1). Fresh mass (FM) was high in the first 2 months of aestivation, and dropped thereafter until September. The decline of FM was linear over the period of aestivation, but with a low rate ($FM = 0.205 - 0.0061 \times \text{duration of aestivation}$; $F_{1,26} = 34.32$, $P = 0.0001$, $r^2 = 0.570$). Subsequently, FM increased and remained rather constant. However, FM differences among months were not biologically substantial. Even in August (the month with the lowest FM), FM was 87.2% of that measured in May (the month with the higher mean FM). Similarly, changes in FM between the two periods were not considerable, but on average it was higher during the active period. However, there was an average difference of only 3% between the two periods, and no statistically significant difference between them ($F_{1,69} = 3.87$, $P = 0.053$).

DISCUSSION

Animals exhibit morphological, behavioural, physiological and biochemical adaptations for life in stressful environments. The crucial elements for survival during aestivation, particularly for pulmonate land snails, are water retention and sufficient energy reserves (Storey, 2002). Our results on behaviour, biochemical content, enzyme activity and fresh mass of the studied population of *A. caerulea*, indicate that several adaptations are in effect, especially before and during aestivation. The fact that mortality is not significantly higher during aestivation provides a strong indication of this.

For land snails, at the morphological level, shell and apertural modifications are considered to be of great importance (Machin, 1975). *Albinaria caerulea* has a thick, white shell and exhibits apertural characteristics (strengthened and reflected lips, internal lamellae and door-like clausilium) that help in reducing water loss, especially when aestivation occurs in locations exposed to sun (Giokas, Mylonas & Sotiropoulos, 2000). In snails, a white shell may reflect 90–95% of visible and infrared wavelengths (Yom-Tov, 1971c). In *Albinaria*, shell thickness is a major factor that contributes to higher survival during aestivation (Giokas

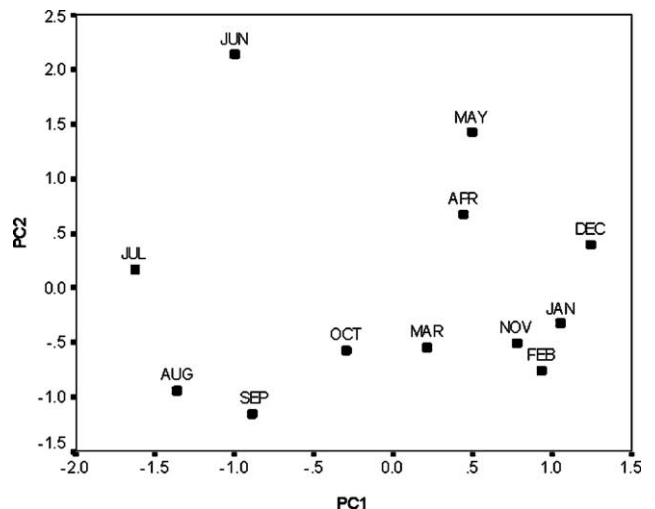


Figure 2. Principal component analysis ordination plot of the sampling months.

et al., 2000). Strengthened and reflected lips help give a closer sealing of the aperture to the substratum (Machin, 1975), and internal lamellae and door-like clausilium may reduce desiccation (Warburg, 1972; Machin, 1975; Gittenberger & Schilthuisen, 1996; Giokas *et al.*, 2000). In addition to these structural adaptations, epiphragm secretion is related to the reduction of water loss in land snails (Machin, 1975; Barnhart, 1983; Cook, 2001) including *Albinaria* (Giokas & Mylonas, 2002; Giokas *et al.*, 2000). *Albinaria caerulea* forms an extremely thick multi-layered epiphragm and adheres firmly on the rock surface during aestivation (see also Giokas & Mylonas, 2002).

At the behavioural level, land snails usually seek sheltered locations during aestivation in order to conserve body water, minimize their exposure and avoid predators. In addition, water loss is reduced by sealing the shell aperture directly to the substratum or the shell of another animal, by more pronounced aggregating behaviour, and in the case of aestivation in exposed locations, by aestivating just above the ground so as to encounter lower temperatures (Machin, 1975). We have identified all these behavioural characteristics in *A. caerulea*. High aggregation in exposed locations (rock surfaces), or in sheltered places (crevices, shrubs) is typical for this population, and also for other *Albinaria* species (Giokas & Mylonas, 2002). In our study we also found greater aggregation during aestivation. Clustering may offer an effective isolation from environmental conditions since in this way the exposed total surface area is decreased and a more humid micro-environment obtained, resulting in a lower water vapour pressure gradient to the environment and, therefore, a lower rate of water loss (Lazaridou-Dimitriadou & Deguzan, 1981; Prior, 1985; Arad & Avivi, 1998). We also found that, at the population level, there is a synchronization in epiphragm formation.

At the physiological and biochemical levels, aestivating snails also show adaptations that retard water loss during dormancy. Because water is lost during breathing and also across the skin/epithelium, snails normally enter aestivation with large reserves of body water that can be drawn upon to keep tissues hydrated (Storey, 2002). In our study we measured the highest level of fresh mass in May, the first month of aestivation, suggesting that *A. caerulea* entered aestivation with high water reserves. In *A. caerulea* another element of adaptation during aestivation is the remarkably low level of water loss during aestivation. We found that even though fresh mass exhibited a decrease during aestivation and an increase during the active season, differences between these periods were not significant. Furthermore, the rate of decline of fresh mass during aestivation was low. However, because these data derive from a single year, it is difficult to decide whether the decrease of fresh mass in January is an irregularity or a recurring event. Warburg (1972) obtained similar results studying various Israeli clausiliid species. Evaporative water loss during breathing is also minimized by apnoeic breathing patterns (Barnhart & McMahon, 1987). In addition, the establishment of high concentrations of solutes elevates the osmolarity of body fluids and slows down water loss. Various snail species use urea for this purpose (Rees & Hand, 1993; Arad, 2001). Arad & 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 & Avivi (1998) suggested that snails regulate their water budget through metabolic pathways by oxidation of storage substrates that contribute extra metabolic water. Steinberger, Grossman & Dubinsky (1982) have estimated that in *Sphincterochila prophetarum* (Bouguignat, 1852) the contribution of metabolic water to total water content is about 8% during aestivation, and partly compensates for the summer water loss. However, even though we did not find a significant decrease of dry mass during aestivation, we cannot refute the above suggestion that storage substrates provide metabolic water during aestivation.

On the other hand, conservation of fuel reserves, resulting from metabolic rate depression, is essential for survival during aestivation. 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). 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 of digestion and movement, 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 oxy-conforming species (Storey, 2002). However, a significant part 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 & Storey, 1989; Storey & Storey, 1990; Rees & Hand, 1991; Guppy, Fuery & Flanagan, 1994). However, there are other influences on metabolism during aestivation such as maturation of reproductive tissues requiring metabolic adjustments in selected tissues (Storey, 2002), possibly also acting in *Albinaria* where maturation of genitalia continues during aestivation (Schilthuisen, 1994; Giokas, 1996).

The laying down of large reserves of endogenous fuels precedes aestivation, and supports metabolic fuel supply during dormancy (Storey, 2002). In our study we identified a pattern of use of metabolic fuels typical for land snails. We found that lipids and sugars are at their highest concentration during the first month of aestivation. However, sugars were rapidly used up and lipids decreased only after the second month of aestivation. The use of proteins changes over time, being low during the early weeks of aestivation and then increasing; proteins declined only after the third month of aestivation, as the water potential declines and possibly the demand for urea synthesis increases. Catabolism of carbohydrates seems to be of major importance for aestivating land snails (Livingstone & de Zwaan, 1983). Rees & Hand (1993) have also found that polysaccharides were the primary metabolic fuel for the first 2–4 months of a 7-month aestivation period, and when it was depleted net protein catabolism began, and a low rate of lipid catabolism was maintained throughout.

Metabolism in aestivating land snails is aerobic, with minimal employment of anaerobic pathways (Rees & Hand, 1990; Brooks & Storey, 1997; Michaelidis, 2002). Glycolysis is down-regulated and internal oxygen levels are regulated, 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 & Storey, 1997; Withers, Pedler & Guppy, 1997; Michaelidis, 2002). Usually, aestivating land snails do not show lactate accumulation and completely oxidize carbohydrate (Churchill & Storey, 1989; Brooks & Storey, 1997). However, in our study on *A. caerulea*, we have found lactate and LDH accumulation to take place at least during the first months of aestivation. This outcome contrasts with most similar studies on aestivating land snails (see Brooks & Storey, 1997). Nevertheless, some evidence indicates that, at certain times during aestivation, energy production in land snails may also be based on anaerobic processes (Wieser & Wright, 1978, 1979; Michaelidis, Pallidou & Vakouftsi, 1999). Terrestrial snails may find themselves in oxygen-free condition, e.g. when they burrow deep in the ground (Von Brand, 1944). Moreover, 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, Taylor & Shkolnik, 1971). Furthermore, Wieser & 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. The revealed pattern of LDH activity indicates that *A. caerulea* is perhaps capable of lactate oxidation when the

concentration of this metabolite reaches a critical value (see also Wieser, 1978). Normally LDH is partly inactivated by being bound to cellular structures. According to Wieser (1978) the rise in lactate concentration leads to modifications of the intracellular environment that in turn trigger more LDH molecules to be mobilized from their inactive state. Despite the ability to oxidize the metabolite, the balance of the reaction catalysed by LDH is such that lactate accumulates. However, when changes in the intracellular environment become critical, there is an activation of LDH and acceleration of net removal of lactate from the tissues. The significant cross-correlation between LDH activity and lactate content that we identified conforms to that hypothesis. The disappearance of lactate possibly introduces a second phase of anaerobic metabolism, involving the formation of succinate, and perhaps also of other end-products (Wieser, 1978; Michaelidis *et al.*, 1999). However, this subject is controversial (Michaelidis *et al.*, 1999), and it is possible that other mechanisms are also involved, but the accumulation of lactate may be the factor that starts the whole sequence of events. The revealed correlation between the seasonal activity of LDH and the climatic factors examined, especially with temperature, has been also revealed in studies on *Helix pomatia* Linnaeus, 1758 (Wieser & Wright, 1979), and may indicate compensatory acclimation of this energy-associated enzyme. However, more work is needed in order to explain the unexpected rise of lactate and LDH during aestivation in *Albinaria*.

According to Cook (2001) there are probably 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. Secondly, this preparation may be overridden by events and animals may be forced into a dormant state by prolonged dehydration, high temperatures, etc. *Albinaria caerulea* starts to aestivate before the end of the rainfalls, and aestivation does not cease before the actual start of rainy season. It is of adaptive advantage for pulmonates not to become active in the summer in response to short-term falls in temperature or occasional heavy rains. Cook (2001), summarizing 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 did not find any correlation of biochemical variables (except LDH) with climatic conditions. Yet, interestingly, the biochemical variables, taken as a whole, are sufficient to distinguish the periods of aestivation and activity (Fig. 2). Previous studies on *Albinaria* species (Giokas & Mylonas, 2002) have shown that the start of aestivation is primarily controlled by an endogenous component. Perhaps, changes in the biochemical composition are a by-product of this component and not a direct response to climatic conditions. Possibly this is an advantage in the climatically unpredictable, but normally semi-arid Mediterranean-type ecosystems. 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 upon activation (Storey, 2002). We have found that aestivating *Albinaria* become activated rapidly (see also Giokas & Mylonas, 2002). Field observations and activation laboratory experiments in *Albinaria* have shown that mean activation time is short (100–300 min), with minimal values less than 10 min (Giokas, 1996). Furthermore, in the present study we have found that lipids, proteins and sugars tend to accumulate rapidly after activation. In particular, the rapid accumulation of lipids and proteins after activation that we observed is possibly associated with the reproductive cycle of *Albinaria*, and more specifically with egg production. Thus, as Storey (2002) suggested, aestivation is not dormancy, where organisms are unresponsive or must undergo

major metabolic/developmental changes in order to become active. Therefore, mechanisms of metabolic suppression in aestivators must (a) be rapidly reversible, and (b) require very little metabolic reorganization.

In conclusion, our study showed that *A. caerulea* displayed significant seasonal changes in biochemical composition and enzyme activity, especially during aestivation. Moreover, morphological, behavioural, physiological and metabolic adaptations seem to ensure sufficient water and fuel reserves, and thus relatively low mortality during aestivation. Fresh-mass differences between the aestivation and active period were not significant, and the rate of fresh-mass decline during aestivation was low. Sugars were catabolized early in aestivation, and proteins and lipids seem to be the primary metabolic substrates after sugar reserves were depleted. Accumulation of lactate and LDH suggests that anaerobic pathways can also possibly supply additional energy, especially in the early phase of aestivation. In addition, *A. caerulea* shows a series of morphological (thick white shell, reflected lips, thick epiphragm) and behavioural (high aggregation) characteristics that support survival during adverse climatic conditions by preventing desiccation. These results, in conjunction with the absence of elevated mortality during aestivation, indicate that the study population of *Albinaria* is well adapted to dry conditions, and helps us understand why this genus prevails in semi-arid environments in southern Greece. Even though data relate to a single year, changes are assumed to be general because fluctuations do not correlate with weather conditions. However, it is not clear whether some of our findings, such as the decrease of fresh mass in January, will be repeated in subsequent years, and additional work is needed. Further comparative studies on the ecology and physiology of other *Albinaria* populations displaying significant morphological, behavioural and genetic differentiation from ours, would help the interpretation of functional capacities within an environmental context, and our understanding of the action of natural selection, especially if we consider physiological changes as trade-offs in the currency of fitness.

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