CAN THE ENVIRONMENT INDUCE INTRA-VARIETY CHANGES OF *Helix pomatia* CONCHOLOGICAL FEATURES?

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Abstract. Inter- and intra-specific genetically and phenotypically determined variations in snail morphological features are well documented. The same may be true even within the same species variety. The snails (*Helix pomatia* var. *Banatica*) were collected from two distinct sites, placed 100 km one from another and characterized by different climatic conditions (rainfall level, altitude, annual average temperature, and subtype of temperate climate): Oraviţa and Timişoara area. Using bi - and tridimensional data processing, statistical, and biochemical analyses we assessed the cumulated actions of environmental factors on intra - variety changes of shell morphological features in relation to origin area. Formula proposed for shell tri-dimensional processing (shell volume) provided a reliable and faster method to assess variations among shell height, width, and depth than the multiple analyses of each feature apart. Similarly, aperture bi-dimensional processing (aperture area) successfully replaced the separated statistical analyses of aperture height and depth. Most biometric features, excepting shell thickness, presented a linked and proportional pattern among themselves. In addition, the sense of these relationships (direct or converse proportional) for shell height, width, and depth seemed to vary depending on location. We demonstrated that shell volume and aperture area tend to be larger in areas with higher annual rainfall level, altitude, and mean average temperature. Oppositely, shell thickness displayed a conversed relationship, possibly related to higher calcium content registered for the same population. Overall, our findings suggest that within the same species variety gastropod shells follow a genetic pattern of growing that allow the preservation of their geometric shape irrespective of the environmental conditions.

Keywords: *Helix pomatia*, shell, biometry, geographical variation.

INTRODUCTION

From an evolutionary point of view, gastropods are one of the most versatile groups of mollusks, characterized by an extraordinary biodiversity and capacity to adapt to various environmental conditions [9, 20]. Biometric variations of land snail shells have been extensively documented, but their major determinants are poorly understood [4, 25]. Genetically determined variations in shell shape and size are well known [13, 22], but at the same time, the environment plays a key role in shell development [4, 11, 37]. Thus, distance between two populations can induce shell size variability within species [26]. On the other hand, some land snails (e.g., *Arianta arbustorum*) showed a negative correlation of shell size with altitude [5], although no correlations have also been demonstrated to several species (e.g., *Albinaria idaea*) [38]. Some studies associated larger snails with more humid conditions [31] whereas others considered that they are more probably associated with higher tolerance to freezing [1]. In addition, the shell thickness seemed to be associated with the areas with low rainfalls [32] while the aperture area tended to be smaller under drier conditions [18]. As a result, shell shape variation within the same species is the subject of many studies [2, 14], but no relevant studies have been carried out within the same land snail variety.

Thus, our study aimed to determine whether the cumulated actions of environmental factors (altitude, rainfall level, temperature, climate) can induce shell features variations within the same variety of land snail (*Helix pomatia var. banatica*). The first step was to assess which of the shell main features displayed a linked and proportional intra-variety pattern among themselves. This task was achieved by analyzing the simple linear correlations among the following shell features: shell height, shell weight, shell depth, aperture height, aperture depth, and shell thickness. Next, we assessed the impact of environmental factors on the shell morphology using data provided by the National Institute of Meteorology and Hydrology and the specialty literature. In addition, we aimed to improve and simplify the statistical analysis of shell features variation. Thus, we tested the reliability of shell volume as accurate tri-dimensional processing of shell length, width, and depth. Similarly, we evaluated the reliability of aperture area as accurate bi-dimensional processing of aperture height and width.

MATERIALS AND METHODS

Snail gathering

The snail systematic classification (*Helix pomatia* var. *banatica* Kimakowicz, 1890) was based on the information related to the shell morphology (shape, appearance, and color) provided by Grossu [20]. A hardened and turned-out aperture lip was considered as a sign of sexual maturity. As shown in Table 1, the locations were chosen in relation to their different rainfall level, altitude, annual average temperature, and subtype of temperate climate. The distance between the collection sites was 100 km: Oraviţa (Caraş-Severin county; Latitude: 45.2°N; Longitude: 21.41°E) and Green Forest, nearby Timisoara (Timis county; Latitude: 45.47°N; Longitude: 21.17°E). Since the soil horizons in the Green Forest area were undulated, with bumpy terrain, the assay method was developed based on the random-walk technique [27] aiming to provide an accurate and statistically significant sampling of twenty Roman snails (*Helix pomatia*) for each location.

Snails were anaesthetized using a solution made up of 25% ethanol and 75% distilled water [20].

Biometric features determination

The major shell conchological features (height, width, depth, thickness) were established according to standard methods used in malacology [23]. First, the shell was positioned with the apex upwards and the aperture up to the operator (Fig. 1A). The shell height (h_s) was measured from the apex to the shell lowest point. The aperture height (h_a) was estimated as the aperture circumscribed circle diameter whereas the aperture width (w_a) was determined as the largest diameter perpendicular to the aperture height (Fig. 1B). Then, the shell was positioned with the apex up to the operator and the apex-umbilicus axis parallel to the ground (Fig. 1C). The shell width (w_s), also known as the large diameter, was estimated as the length of the rectangle circumscribed to the shell greatest section. The shell depth (d_s), or the small diameter, was considered as the width of this rectangle. Next, the shell thickness (t_s) was measured near the junction between the aperture upper part and the body whorl. To provide accurate results, each measurement was repeated three times and the mean value was taken into account.

Table 1. Snail area of origin, monthly mean rainfall level (X ± SE), annual average temperature (AAT), altitude, and climate type for the two locations.

Environmental Factor / Origin area	Monthly mean rainfall level (mm)	Annual average temperature (ºC)	Altitude (m)
Oravita	74.58 ± 17.68	11.8	309
	Temperate continental climate		
Green Forest	56.07 ± 11.89	10.6	91
	Temperate continental climate with Mediterranean influences		

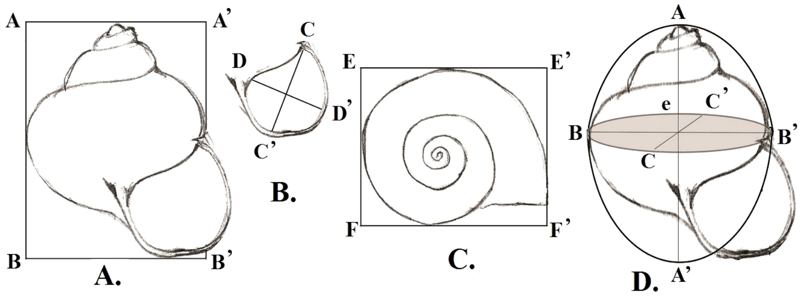

Figure 1. A. - Shell positioned with the apex upwards and the aperture up to the operator. Abbreviations: AB, A\`B\`, shell height; AA\`, BB\`, shell width/large diameter. **B.** - Aperture morphological features estimation. Abbreviations: CC\`, aperture height; DD\`, aperture width. **C.** - Shell positioned with the apex up to the operator. Abbreviations: EE\`, FF\`, shell width; EF, EF\`, shell depth. **D.** - Shell volume estimation. Abbreviations: AA\`, shell height; BB\`, shell width, CC\`, shell depth. **e** - ellipsoid circumscribed by the shell height and width; e, ellipse circumscribed by the shell width and depth.

To calculate its volume, the shell was considered a scalene ellipsoid (Fig. 1D). If the snail shell is longitudinally sectioned, the largest area will be the ellipsoid circumscribed by the shell height and width (***E***). Similarly, if the snail shell is transversely sectioned, the largest area will be the ellipse circumscribed by the shell width and depth (***e***). By circumrotating the ellipsoid ***E*** around the ***e*** ellipse, the shell volume (V_s) can be assessed according to the formula:

$$V_s = \frac{\pi \cdot h_s \cdot w_s \cdot d_s}{2000}$$

Next, the aperture area (A_a) was estimated according to the formula:

$$A_a = \frac{\pi \cdot h_a \cdot w_a}{1000}$$

It must be mentioned that both formulae used conchological features expressed in mm and processed them by-dimensional in cm^2 and three-dimensional in cm^3.

Climatic data

To allow a comprehensive understanding of relationships between environment and conchological features several parameters were counted in this purpose for both locations (Table 1), as follow: subtype of temperate climate, annual average temperature (AAT), annual rainfall level (AAR), monthly average rainfall (MAR) from 1995 to 2005, and altitude. These data were collected from the literature [29, 36] and from the historical meteorological database available at The National Institute of Meteorology and Hydrology. Since MAR variation is extremely important for *H. pomatia* life cycle considering its preponderant herbivorous diet [23], this parameter was estimated by statistical analysis, whereas the other factors were used as simple mean values according to the climatic and geographical recordings.

Establishing the shell calcium content

For each originating location, 10 snail shells were dried, grinded, and mixed together. Next, the samples were kept in abeyance for two days. One gram/sample was then put in each of two test tubes. Then, 6N HCl solution was added in order to avoid excessive foaming. The tubes were boiled (2 h), their content was filtered and then put in a calibrated flask (100 ml), filled with deionized water. The calcium was determined by calcium-EDTA titration in strong alcaline medium, using murexide as indicator [38].

Statistical analysis

Biometric parameters were analyzed by descriptive, parametric, and nonparametric statistical tests. First, we assessed the distribution normality (Anderson-Darling test) for the main biometric features: shell height, shell width, shell depth, shell thickness, aperture height, aperture width, and shell thickness. Next, the canonical correlation analysis (CCA) was conducted to find the relationships existing among these features. Then, we determined the biometric features variations between the two snail populations (F test, two-tailed, df = 2, 36). After that, we assessed the origin influence on the shell height, width, length, volume, and thickness, on one hand and on the aperture height, width, and area, on other hand (T test, one-tailed, df = 2, 36). To provide an assertive interpretation of these conchological features variation we used the one-tailed T test that allows the unidirectional and targeted processing of data as related to their statistical significance.

Second, the annual rainfall levels (MAR) were analyzed regarding their distribution normality (Anderson-Darling test). Finally, we assessed the differences between the two habitats from MAR variation point of view by using the Mann-Whitney test (two-tailed, df = 2, 22).

Data are presented as mean +/- SE. A p-value <0.05 was considered significant.

RESULTS

Do shell features present a linked and proportional pattern among themselves?

The Anderson-Darling test proved a normal distribution for all the biometric parameters taken into account (at least $p < 0.05$). As shown in Table 2, descriptive statistics revealed that concerning either the shell height, width, and depth or the aperture height, the Oravita population was more homogeneous than the Green Forest one. Oppositely, regardless of location, both the aperture width and the shell thickness were medium-sized variability features. However, the shell parameters in both populations displayed close values to the ones reported in Romanian literature for *Helix pomatia* var. *banatica*: shell height: 43 mm; shell length: 51mm; shell width: 40 mm; aperture height: 33-34 mm [20].

Table 2. Biometric features means and ranges depending on location.

Origin area / Biometric parameter	Oravita area		Green Forest area	
	X ± SE	Range	X ± SE	Range
Shell height (mm)	38.95 ± 3.33	32.20 – 46.00	34.62 ± 4.67	24.80 – 43.80
Shell width (mm)	38.20 ± 3.15	28.70 – 43.00	33.20 ± 3.93	26.40 – 40.80
Shell depth (mm)	32.80 ± 2.79	26.50 – 37.60	29.78 ± 3.71	23.20 – 37.40
Shell thickness (mm)	0.65 ± 0.10	0.50 – 0.80	0.72 ± 0.10	0.50 – 0.90
Shell volume (cm^3)	77.16 ± 17.03	40.62 – 109.68	57.04 ± 20.67	24.64 –104.93
Aperture width (mm)	23.21 ± 2.64	19.00 – 28.50	20.01 ± 2.56	14.80 – 24.60
Aperture height (mm)	27.36 ± 1.68	24.60 – 31.20	25.24 ± 2.67	20.10 – 30.80
Aperture area (cm^2)	2.00 ± 0.32	1.46 – 2.71	1.60 ± 0.12	0.93 – 2.28

Note: X ± SE (mean ± standard error of the average).

As shown in Table 3, canonical correlation analysis (CCA) for Oravita population revealed significant positive linear relationships between shell height and the other main biometric parameters: shell width, shell depth, aperture height, aperture width, and shell thickness. Positive correlations were also found between shell width and both shell depth and aperture width. Oppositely, shell width displayed a weak correlation with aperture height. In addition, no correlations were identified between shell width and thickness. Shell depth was positively correlated with aperture height, aperture width but not with shell thickness. Similarly, aperture height was strongly correlated with both aperture width and shell thickness. Conversely, we found a weak relationship between aperture width and shell thickness. In addition, F test demonstrated that there are no significant differences regarding the main biometric features variation for the two *H. pomatia* populations: shell height ($F = 0.50$, $p = 0.16$), shell width ($F = 0.64$, $p = 0.35$), shell depth ($F = 0.56$, $p = 0.23$), shell volume ($F = 1.06$, $p = 0.17$), aperture height ($F = 0.50$, $p = 0.16$), aperture depth ($F = 1.06$, $p = 0.89$), aperture area ($F = 0.84$, $p = 0.72$), and shell thickness ($F = 0.92$, $p = 0.86$).

Intra-variety changes of shell morphology

The annual rainfall level presented a higher monthly variability in the Oravita area (Table 1). Anderson-Darling test proved an abnormal distribution for this parameter, regardless of location (Anderson-Darling test, $p > 0.05$). In addition, significant differences were revealed between the monthly rainfall level (MMA) registered in the Oravita area and in the Green Forest area ($U = 13.00$, $p = 0.001$). The calcium content (Ca^{2+}) was higher for snails living in the Green Forest area than for those sampled from the Oravita area: 37.36% vs. 37.19%. As shown in Table 2, Student`s T test revealed that shell height was significantly higher ($t = 3.28$, $p < 0.01$), wider ($t = 3.47$, $p < 0.01$), and deeper ($t = 2.85$, $p < 0.01$) for the snails living in areas with higher rainfall level, mean temperature, and altitude (Oraviţa population). A similar trend was also registered for both aperture width (t = 2.88, p < 0.01) and depth (t = 3.79, p < 0.001). Oppositely, snails inhabiting Green forest area displayed significantly thicker shells (t = - 2.356, p < 0.05).

The shell volume was significantly higher (t = 3.27, p < 0.01) for the Oravita population as compared to the Green Forest samples. Similarly, the aperture area was also significantly higher (t = 3.59, p = 0.001) for the same snail population. The main biometric features that influence the shell volume are the shell height, width, and depth. For the Oravita population, the relationship among them was most precisely estimated by using the formula (R^2 = 0.29):

$$h_s = A^{(B/w_s + C \times d_s)}$$

where $A \approx 0.23$, $B \approx -0.99$, and C ≈ 0.16. On the other hand, in the Green Forest area, these relationships were assessed with the same precision (R^2 = 0.29) by using another formula:

$$h_s = A \times d_s^{(B/w_s)}$$

where A ≈ 0.87, B ≈ - 0.10, C ≈ 0.16.

Table 3. CCA analyses of the main shell biometric features (n =20/group).

Biometric feature	Shell height	Shell width	Shell depth	Shell thickness	Aperture height	Aperture width
Oravita area						
Shell height	-	R = 0.73 p < 0.0001	R = 0.68 p < 0.0001	R = 0.51 p < 0.05	R = 0.70 p < 0.0001	R = 0.60 p < 0.01
Shell width	R = 0.73 p < 0.0001	-	R = 0.79 p < 0.0001	R = 0.36 p > 0.05	R = 0.47 p < 0.05	R = 0.74 p < 0.0001
Shell depth	R = 0.68 p < 0.0001	R = 0.79 p < 0.0001	-	R = 0.34 p > 0.05	R = 0.60 p < 0.01	R = 0.80 p < 0.001
Shell thickness	R = 0.51 p < 0.05	R = 0.36 p > 0.05	R = 0.34 p > 0.05	-	R = 0.08 p > 0.05	R = 0.49 p < 0.05
Aperture height	R = 0.70 p < 0.0001	R = 0.47 p < 0.05	R = 0.60 p < 0.01	R = 0.08 p > 0.05	-	R = 0.71 p < 0.0001
Aperture width	R = 0.60 p < 0.01	R = 0.74 p < 0.0001	R = 0.80 p < 0.001	R = 0.49 p < 0.05	R = 0.71 p < 0.0001	-
Green Forest area						
Shell height	-	R = 0.87 p < 0.0001	R = 0.93 p < 0.0001	R = 0.12 p < 0.05	R = 0.84 p < 0.0001	R = 0.68 p < 0.001
Shell width	R = 0.87 p < 0.0001	-	R = 0.89 p < 0.0001	R = 0.06 p > 0.05	R = 0.67 p < 0.01	R = 0.92 p < 0.0001
Shell depth	R = 0.93 p < 0.0001	R = 0.89 p < 0.0001	-	R = 0.07 p > 0.05	R = 0.90 p < 0.0001	R = 0.75 p < 0.001
Shell thickness	R = 0.12 p < 0.05	R = 0.06 p > 0.05	R = 0.07 p > 0.05	-	R = 0.08 p > 0.05	R = 0.55 p > 0.05
Aperture height	R = 0.84 p < 0.0001	R = 0.67 P < 0.01	R = 0.90 p < 0.0001	R = 0.08 p > 0.05	-	R = 0.77 p < 0.0001
Aperture width	R = 0.68 p < 0.001	R = 0.92 p < 0.0001	R = 0.75 p < 0.001	R = 0.55 p > 0.05	R = 0.55 p > 0.05	-

Note: R (correlation coefficient)

DISCUSSIONS

Do shell features present a linked and proportional pattern among themselves?

A gastropod shell generally exhibits a logarithmic spiral growth in a clockwise direction of coiling around a single axis [12] whereas their growing pattern follows the Fibonacci sequence pattern [33]. On the same lines, it is also known that to ensure an optimum shape for shell balance on the substrates, terrestrial gastropods display a bimodal distribution [30]. Our results materialized through highlighting the presence of strong correlations among most major conchological features and the absence of significant differences among them pointed to a linked and proportional intra-variety pattern of shell biometry variation for *Helix pomatia* var. *Banatica.* Contextually, all these data suggest the existence of a genetic background that allows the snails to grow in such a way that their body remains more or less the same size in relation to shell, it fits into the big end of the shell, and the shell does not become too heavy to drag around.

Intra-variety changes of shell morphology

Shell volume is usually determined by water displacement or by filling them with water using a calibrated syringe [20, 23]. Other authors [6, 16] used formulas based on shell height (h_s) and width (w_s) although these conchological features are rarely equal as proved by our measurements conducted on *H. pomatia*. As a result, we assume that this formula most important asset is that it allows a quick and significant three-dimensional processing of shell features through the shell volume (cm^3), as our study demonstrated. Similarly, the bi- dimensional processing of aperture features as aperture area (cm^2) represents a reliable and faster method to assess the variations of aperture width and depth among snail populations than the repeated comparative analysis of differences between these populations for each feature apart.

Well known geographical variations of shell biometry are most probable based on different climatic conditions [38] and life-history tactics [26]. This process results in changes of ratio between major conchological features such shell height/shell diameter

[10] or aperture height/aperture width [17]. Our data pointed that these variations also occur within the same species variety. Thus, higher shell height/shell width ratio for GH and GA snails (1.04 vs. 1.01). Also, similar results are found for the aperture features ratio (1.25 vs. 1.17). In addition, the bifactorial exponential regression established for the Oravita population indicated a direct proportional relationship between the shell height and width and a reversed one between the shell height and depth. In contrast, for the snails living in the Green Forest area the shell height seemed to be directly proportional with depth and reversed proportional with height. As a result, we concluded that cumulated actions of environmental (altitude, rainfall level, temperature, climate) factors induce shell features variations within *Helix pomatia* var. *Banatica*.

Several studies associated larger snails with more humid conditions. The effect may be inductive (direct) or selective, but the mechanism is not well documented [31] although the relative aperture area was suggested to decrease as a result of decreased water loss provided by selection for smaller whorl cross-sectional area [18]. Our research proved the viability of these findings even within the same species variety. Thus, the shell volume and the aperture area were significantly higher for the snails living in the Oraviţa area whereas the annual rainfall level was lower in the Green Forest area (631 mm vs. 895 mm). On other hand, for *H. aspersa* increased shell thickness reduced water loss and was considered a defense mechanism against drought [24]. Contextually, this study revealed that shell wall was significantly thicker for the snails living in areas with lower annual rainfall level (Green Forest population). It was explained for *H. pomatia* that thicker shells probably increase the life expectancy and enable the snail to successfully pass through a series of poor breeding seasons [32]. Based on shell growing mechanism [34] we suggested that a longer life involves a thicker shell, a thicker hipostracum, and thus a higher content of calcium as found to snails inhabiting the Green Forest area. Nonetheless, in order to derive some relevant conclusions regarding the precise relationships existing between shell calcium content and its thickness further studies need to take into account the soil chemical composition since it is known that land snails abundance is associated to calcareous soils [19].

Since shell polymorphism is a problem with many potential solutions [22] we assume that these variations are the result of environmental factors cumulated actions (e.g., meteorological conditions, soil composition, pollution degree, anthropization level, altitude, etc.) than the phenotypic impact of a single one. Among factors that we did not assess in the current study, but may be contributing to the environmental effect on the identified phenotypes, is the potential presence of heavy metals in the air and soil. The Oravita area is well-known in Romania for the presence of disaffected uranium mines in the area Ciudanoviţa-Lişava [21] whereas the amount of led exhausted into the atmosphere that increased faster over the last two decades following the accelerated industrial development affects snail metabolism [15]. The shell height tended to be higher for specimens collected nearby the nuclear plants [36], whereas lead accumulation decreased shell mass in different snail species [7, 8, 35]. Uranium was shown to have cytotoxic, genotoxic, teratogenic, and carcinogenic effects in animal studies [3, 28] but prolonged exposure to heavy metal contamination may lead to selection of metal-tolerant phenotypes of snails [7]. Future studies will elucidate whether this is indeed the case.

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