

Stable or plastic body shape? *Emys orbicularis* hatchlings-juveniles growth patterns under different ecological conditions

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Abstract. To test if habitat could shape shell features of freshwater turtles starting from birth, we tested hatchlings of *Emys orbicularis*, randomly chosen from four clutches of the same locality, under two treatments, stagnant (pond habitat) vs lotic (river habitat) water regimes. Due to mortality at the beginning of the experiment, we finally considered only two clutches. Turtle shells were characterized initially at hatching and then after one year of treatment by means of traditional and geometric morphometrics, and phenotypic trajectories analyses were conducted on both types of data. Carapace growth was isometric both in proportion and shape, while plastron showed an allometric pattern: length increased more than width, while the contour showed an expansion at the rear and a compression of the front. The hydrodynamic conditions during growth did not seem to affect the phenotypic trajectories of the shell proportions or the plastron. On the contrary, a significant effect was found on the phenotypic trajectory of the carapace: the size component (but not the angle one) was positively influenced by the "lotic" treatment, suggesting a different growth rate between the two environmental conditions.

Key words: *Emys orbicularis*, hatchlings, contrasted habitats, growth patterns, body shape, phenotypic variation.

Introduction

The bony and epidermal carapace of Chelonians represents a structure usually considered less phenotypically plastic than soft parts. However, research showed that habitat features and feeding habits play a significant role in modifying carapace shape in terrestrial and aquatic species (Claude et al. 2003). Mechanical implications in sexual dimorphism have also been found relevant using shape analyses (Vega & Stayton 2011). Finally, fecundity selection in females and sexual selection in males have been suggested as the main sources of phenotypic variation in freshwater turtles (Bonnet et al. 2010). To date, there is limited research background for the dynamics of the shell growth and shape of freshwater turtles, especially during their first year, a key period that likely determines the final shape of the turtle's shell, and on the habitat conditions that might influence this process.

The speed of running water is a critical feature of the habitat in which aquatic species live and it characterizes many aspects of their biology. A strong relationship between stream speed and or-

ganism morphology has been often underlined in a wide range of taxa: plants (Puijalon & Bornette 2004, Boller & Carrington 2006, Stewart 2008), invertebrates (Marchinko 2003, Holomuzki & Biggs 2006), and vertebrates (Pakkasmaa & Piironen 2001, McGuigan et al. 2003, Peres-Neto & Magnan 2004, Rivera 2008). In most of the cited cases, the variations of shape have been interpreted as of adaptive value: individuals living in fast running waters show morphological characteristics that markedly reduce the strength of resistance to the water, thus improving their body structure. However, these directional adaptations may negatively affect performances in other contexts, for instance in prey-predator interactions or during thermal exchanges (Polo-Cavia et al. 2009).

Many species of fresh water turtles show a body development apparently caused by diet, feeding and habitat (Claude et al. 2003). Recent studies have also suggested that hydrodynamics represents a primary factor influencing intraspecific variation of carapace size and shape (Lubcke & Wilson 2007, Rivera 2008). The friction forces acting on the carapace and plastron increase in intensity equal to the squared difference between the

vectorial velocity of the animal and the water speed. In habitats and environments with relevant stream flows these friction forces are higher than in lentic environments. Body structures with morphological adaptations for hydrodynamism are favoured in reducing the effects of these forces, increasing the energetic efficiency of turtles during movement. In *Pseudemys concinna*, as far as we are aware the only species examined in detail for this pattern, experimental data confirmed such a hypothesis (Rivera 2008).

To date, however, only adult turtles and tortoises have been considered in any experimental, analytical or modelling research. Reaching the adult stage, the carapace and plastron have become rigid and fully calcified structures, thus it is unlikely (however, still to be tested) that environmental factors can induce marked and rapid effects on external features. On the contrary, newborns and juveniles have soft and not fully calcified bony parts that are more readily subjected to phenotypic changes. Surprisingly, it has not been determined yet if a “key period”, if any, does occur in shaping and fixing the final aspect of the shell in Chelonians. Thus, it may be of interest to highlight i) the extent of such a variation, ii) the starting time of the differentiation, and iii) the persistence of the pattern. Nevertheless, turtles and tortoises are long living organisms (Rollinat 1934, Congdon et al. 2003) and long-term experiments of adequate length could take even a researcher's full life span. Despite this, testing if the occurrence of phenotypic patterns may happen just after hatching, may shed light on the timing of variation in shell shape and contribute to the comprehension of the life-history traits and phenotypic plasticity of Chelonians.

As a species model, we chose a common freshwater turtle of the western Palearctic region, the European pond turtle, *Emys orbicularis*, as it is abundant both in Europe (Podloucky 1997, Sillero et al. 2014) and in many Italian areas (Zuffi 2000, Zuffi et al. 2011). Variations of the shell shape in adults of this species were found in contrasting habitats and at different latitudes and altitudes (Zuffi et al. 2007). Therefore, we are interested in understanding the role of hydrodynamism on *E. orbicularis* phenotype, starting at the beginning of turtle's life cycle. Particularly, we aimed at answering the following two questions: i) does carapace shape respond to hydrodynamic conditions during the early stage of growth, immediately after hatching, and if so, how?, and, ii) what role do

environmental stimuli have on the expressed phenotype?

Material and methods

Eleven adult females were captured within a protected, isolated area, in the San Rossore Reserve, within the Regional Nature Park Migliarino San Rossore Massaciuccoli (Pisa, western Tuscany, central Italy). All individuals were captured from pond habitats, during typical ovulation time (late May, 2011). After manual palpation for presence of mature follicles, each female was x-rayed to determine clutch size (Zuffi et al. 2005). Each female was then kept in a common outdoor aqua-terrarium (120 × 100 cm) situated near the capture location, with conditions similar to those at capture location, till the egg laying period (end of June). Food, direct sunlight, and basking places were available *ad libitum* to avoid any competition for food (Rivera & Stayton 2011); water was changed every two days. Out of the 11 captured females, 6 had eggs from x-ray analysis, and 4 effectively laid eggs, producing a total of 17 hatchlings (Table 1). Adults were released shortly after egg laying, and juveniles - at the end of their first year of life.

Once laid, eggs were numbered according to the mother reference code, and incubated till hatching (the incubator was a Brutmaschinen-Janeschitz GmbH, model 400/REP, Germany). Eggs were incubated at slightly lower than 28 °C (from 26.5 to 27.5°C), a temperature that is known to produce 100% phenotypic males in Chelonians (Bull et al. 1982, Pieau et al. 1994, Girondot 1999). Genetic sex determination is still possible, despite a low occurrence of inverted sexes in French *E. orbicularis* (11% in males, 6% in females) (Girondot 1994, Girondot et al. 1994). We have therefore considered this matter as negligible with regard to our experimental sample and design. We have chosen one sex only, in order to reduce any bias due to different growth patterns related to sexual dimorphism. Furthermore, males appear the preferred model, being more active in their ecology than females (Rovero & Chelazzi 1996), and may therefore be featured optimally to respond to natural (and artificial) stimuli related to hydrodynamism, and to a higher degree of environmental stress (Rivera & Stayton 2011).

Hatchlings were reared in two opposite hydrodynamic conditions, simulating “pond” (stagnant water) and “river” (running water) habitat, respectively. Hatchlings from the same clutch were randomly assigned to one of the two treatments to balance parental effect (Table 1). Unfortunately, starting from an initial sample of eight turtles for the “pond” and nine for the “river” treatment, the final sample size was lower (five “pond” and six “river”): the death of two individuals assigned to “pond” treatment led us to exclude their counterparts from the same clutch and assign them to the opposite treatment; further, the occurrence of shell malformation in an individual of the “pond” group led to the exclusion of its two “river” counterparts from the same clutch (Table 1).

The hydrodynamic conditions were simulated

Table 1. Numerical description of clutch size, kin relationship and final sample size for the reared turtles. "Dead" refers to the individuals that died before the end of the year of treatment; "Excluded" represents turtles not used in the analysis because of death, parental balancing, or malformation. "Final sample" = sample actually analysed.

Female (mother reference code)	X-ray clutch size	Laid clutch size	Hatchlings	Assignment		Dead		Excluded		Final sample	
				Pond	River	Pond	River	Pond	River	Pond	River
T	4	2	2	1	1	1	0	1	1	0	0
117	6	6	6	3	3	0	0	0	0	3	3
139	5	3	3	1	2	1	0	1	2	0	0
237	7	6	6	3	3	0	0	1	0	2	3
Total	22	17	17	8	9	2	0	3	3	5	6

through the use of artificial habitats. These were constructed from animal transporters, 45 × 30 × 20 cm (length × width × height) filled with water (level of 5 cm) with a heating element set up at 26–27°C. Light was provided with an ultraviolet and infrared lamp, and a light-darkness cycle set up at 12:12 hours (automated timer). A basking place and a refuge were supplied in each habitat, and food (*Tenebrio* mealworms and *Lumbricus* earthworms) was provided *ad libitum*, with the addition of macrobenthos (Copepoda, Cladocera), and algae taken from the field once a week. Water was changed weekly. For the "river" habitat water was running at ca. 25 cm × sec⁻¹ speed, with a 12:12 hours cycle, stressing the turtles during their normal, diurnal, activity cycle (Zuffi et al. 2011), controlled by an automatic timer; water movement was supplied by a BIODYNAMIS® (Askoll, Italy) water engine. Based on unpublished data, captive adult and juvenile turtles spent most of their night time outside the water, we considered unimportant to experimentally stress turtles during this period. We also stopped lighting and heating during the winter dormancy (December to February), as it naturally happens along the Tuscany coast. The housing room temperature ranged in that period from 3°C to 6°C.

Each hatchling was weighted and measured (according to a standard protocol; Zuffi et al. 1999). Then, to address necessary data for morphometrics analyses and individual recognition we photographed each hatchling, 12 to 48 hours after hatching (the time necessary to allow complete expansion of carapace and plastron; Zuffi, unpubl. data., 2015). Pictures of the ventral (plastron) and the dorsal (carapace) sides were taken keeping the turtle in contact with the table plane, on graph paper, having the camera placed at a distance of 15 cm, orthogonal to the midline of the shell. The same procedures were repeated after one year of treatment, i.e., when calcification was progressed and shape changed a little (see Hirasawa et al. 2013).

Two-steps phenotypic trajectory analysis (Collyer & Adams 2013) was used to compare the growth pattern of the shell under the two treatments. This technique has the advantage of taking into account the dynamic aspect of the path that a specific trait follows during individual growth (Adams & Collyer 2009, Collyer & Adams 2013). The phenotypic trajectory (PT) is represented by the vector that joins the starting and ending points (e.g., at birth

and after one year) in the space of the variables considered. PT is characterised by its length and its direction; consequently, two trajectories may differ because of their lengths (size), the angle between their directions (angle) or both (Collyer & Adams 2007). In the present study, the analysed trait is the shape of the shell. The shape was characterised at birth and after one year, in two different ways. First, we applied traditional morphometrics, which makes use of linear measures (e.g., total length, shell height, etc.), and generates variables inevitably correlated with the size and with each other measure (Claude 2008). Second, we used geometric morphometrics, which removes all size-related information and allows analysing directly the pure shape (Dryden & Mardia 1998).

For the traditional approach, the ratios "carapace length/carapace width", "plastron length/plastron width", and "shell height/carapace length" were used together as shape variables. For the geometric morphometrics approach, 26 homologous landmarks were digitized on carapace images of each turtle (Fig. 1A) and 14 on plastron images (Fig. 1B) using TpsDig2 (Rohlf 2010, available at: <http://life.bio.sunysb.edu/morph/>). The procedure was repeated for each turtle on the images at birth and at the end of the experimental trial (one year). The obtained configurations were symmetrised (Klingenberg et al. 2002) and aligned using a general procrustes superimposition (Rohlf & Slice 1990): these procedures removed left-right asymmetry and all size-related information from the geometry of the object, giving symmetric and scaled configurations of points, whose coordinates become the shape variables (Claude 2008). A principal component analysis (PCA) on the projected coordinates allowed us to obtain the final set of independent shape variables. PTs were calculated as the multivariate differences between the final and the initial configurations of each turtle (Collyer & Adams 2007) using the motion-path method, since the phenotypic trait under study has been measured twice for each individual (Adams & Cherny 2007).

In both geometric and traditional morphometrics, the relations between PT and treatment were analysed using a non-parametric distance-based MANOVA (Anderson 2001, Collyer & Adams 2007): the matrix of the pairwise differences of PT was set as the dependent variables; "treatment" (pond/river) and the size at birth (assessed by log-centroid size for geometric and log-carapace

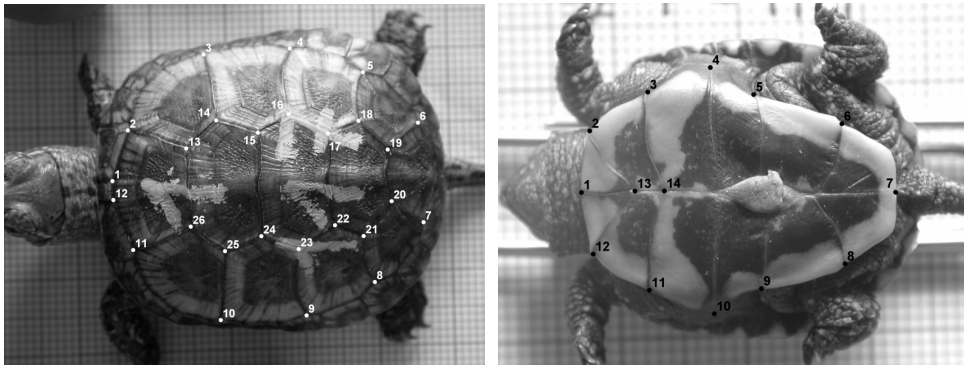


Figure 1. Landmarks on *Emys orbicularis* newborn shell (1a, carapace view; 1b, plastron view)

=length for traditional morphometrics respectively) were set as factors; pseudo-F values were compared to a null distribution obtained via 9999 permutations of records, stratified within "mother", to take into account any possible random effect due to the shared genes among turtles born by the same mother (Oksanen et al. 2013). Distinct MANOVA were conducted for differences in size and angle of the PT.

All the analyses were conducted in R (version 3.0.1; R core Team 2014) using the package "geomorph" for the geometric morphometrics analysis (Adams & Otárola-Castillo 2013), and the function "adonis" of the "vegan" package (Oksanen et al. 2013) for the non-parametric MANOVA.

Results

During the experimental period, the 11 hatchlings grew ca. 19.5 mm in carapace length and 16.1 mm in carapace width (Table 2). The carapace growth appeared isometric, since length and width showed a similar proportional increase (80.3% and 77.8%, respectively), corresponding to a slight variation of the "length/width" ratio, from 1.17 ± 0.04 (mean ± standard deviation) at birth, to 1.19 ± 0.04 after 1 year (paired t test: t = -1.36; d.f. = 11; P

> 0.05). On the contrary, the plastron did not follow an analogous pattern, since its length increased on average by 84.5% (17.9 mm) and its width – by 63.2 % (11.4 mm), with a rise in their ratio from 1.17 ± 0.05 at birth, to 1.32 ± 0.04 after 1 year (paired t test: t = -8.33; d.f. = 11; P < 0.0001). The hydrodynamic conditions during growth did not affect the phenotypic trajectories of shell proportions (Table 3), since both size and angle of the phenotypic vectors were comparable in the two treatments (Fig. 3a).

The general difference in the growth patterns of the carapace and plastron can be appreciated also from the ontogenetic comparison of the carapace and plastron shape obtained by the geometric morphometrics analysis: the carapace showed a stable contour line (Fig. 2a), even though the median scutes reduce their width. On the other hand, the plastron modified its contour in the same direction highlighted by the length/width ratio (Fig. 2b) and showed a lateral expansion of its rear part together with a compression of the anterior one.

Treatment did not affect PT of the plastron (Table 3, Fig. 2c), nor the angular component of

Table 2. Descriptive morphometrics (mean 1SD: standard deviation) of the analysed samples. All linear measures are given in millimetres. N represents sample size.

Treatment	N	carapace			plastron		
		length	width	height	length	width	
Pond	At birth	5	24.15	20.95	12.02	21.13	18.18
			1SD: 1.48	1SD: 0.78	1SD: 0.43	1SD: 1.44	1SD: 1.11
	1-yr old	5	44.60	37.80	18.8	40.20	30.08
			1SD: 4.49	1SD: 2.67	1SD: 1.35	1SD: 4.10	1SD: 2.33
River	At birth	6	24.53	20.63	12.35	21.35	18.10
			1SD: 1.08	1SD: 1.04	1SD: 0.57	1SD: 1.65	1SD: 1.34
	1-yr old	6	43.00	36.02	18.48	37.98	28.92
			1SD: 2.79	1SD: 1.50	1SD: 0.80	1SD: 2.29	1SD: 1.18

Table 3. NP-MANOVA on PT size and angle based on shell proportion (Ratio), carapace shape (Carapace) and plastron shape (Plastron). All models had the same structure and were controlled for hatchling size at birth (not shown). F: value of the observed pseudo-F statistic for the two-levels hydrodynamic treatment (“pond”, “river”); R²: determination coefficient; P: probability of the observed F compared to the distribution obtained by 9999 rows permutations stratified within mother.

Model	PT	F	R ²	P
Ratios	Size	1.155	0.114	0.143
	Angle	0.432	0.046	0.678
Carapace	Size	6.582	0.449	0.047*
	Angle	1.258	0.127	0.121
Plastron	Size	2.457	0.205	0.161
	Angle	1.476	0.134	0.213

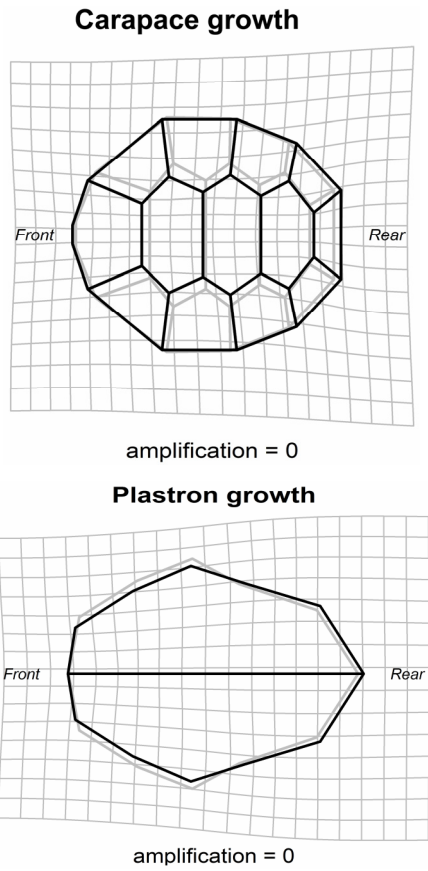


Figure 2. Ontogenetic variation of shell growth (2a, carapace growth; 2b, plastron growth) (grey line = hatchling; black line = 1-yr old turtle).

the carapace PTs (Table 3, Fig. 2b), indicating that it did not influence growth direction. The only statistically supported difference between treatments

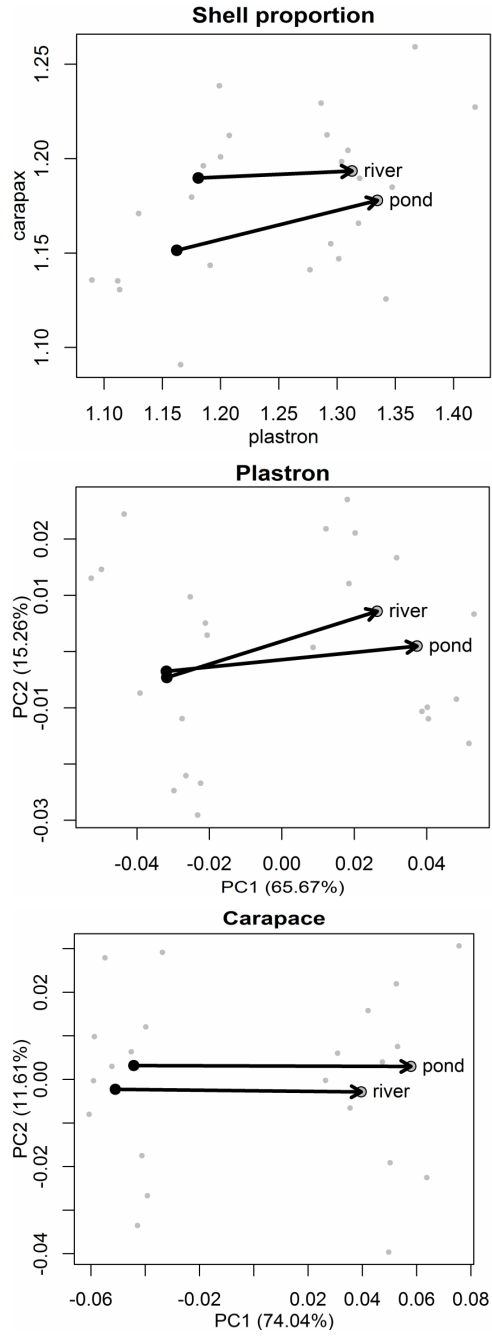


Figure 3. Comparison between the phenotypic trajectories of the two treatments computed on, respectively: shell proportion (3a); shell carapace shape (3b); plastron shape (3c).

concerned the size of the PT, that is, “pond” associated to a longer vector than “river” (Table 3; Fig.

2b). In other words, the carapace of the "pond" treated turtles have prolonged its ontogenetic variation with respect to the carapace of the "river" treated turtles (Fig. 4). Accordingly, the growth rate of the carapace, measured by the relative increment of the centroid size (the mean distance between each landmark and the body centre of mass) revealed that the "pond" treated turtles obtained higher values than the "river" treated ones (Fig. 5), even though the difference was not statistically significant (NP-ANOVA, pseudo- $F=2.24$, $p=0.17$, permutations=9999).

Discussion

The shell shape of *E. orbicularis* significantly varied between the hatchling (also evident from the umbilical scars) and the juvenile stage after the first year of life (Fig. 3). The carapace increased in size, displaying a reduction of the relative size of vertebral scutes, while it preserved its contour line; on the contrary, the plastron modified its profile by narrowing the anterior part and slightly expanding the rear one. The above modification appears to be the result of the change in body proportions (e.g., relative growth of the limbs, the head, the tail, the shell; Carr 1952) and may also be functional for the accommodation of the internal organs, a pattern known in turtles and occurring already at the embryonic stage (Werneburg et al. 2009).

The hydrodynamic conditions can act on the trajectories of phenotypic variation, limited on the carapace shape and the size of the variation, without altering the direction of the PT. "Pond" treated turtles grew as expected, showing a trend towards the typical adult pond turtle shape, with the carapace length almost double the size of the carapace width (Zuffi & Gariboldi 1995). This turtle group was subjected to environmental stimuli similar to those that wild pond turtles experienced in natural conditions (Lebboni & Chelazzi 1998, Fritz 2003, Zuffi et al. 2007). The carapace of "river" treated turtles followed the same growth pathway, but with a shorter trajectory (Fig. 3c). The same trend was indeed replicated also in plastron shape and shell proportion (Fig. 3a, 3b), even though not statistically supported (but the lack of statistical power due to reduced sample size is therefore possible).

In freshwater habitats, forces acting on hard and soft body parts play a similar role in shaping

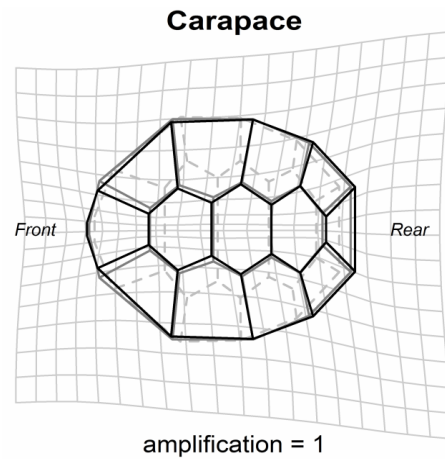


Figure 4. Carapace ontogenetic variation in "pond" vs "river" treated turtles (dotted grey line = hatchlings; grey line = "river"; black line = "pond")

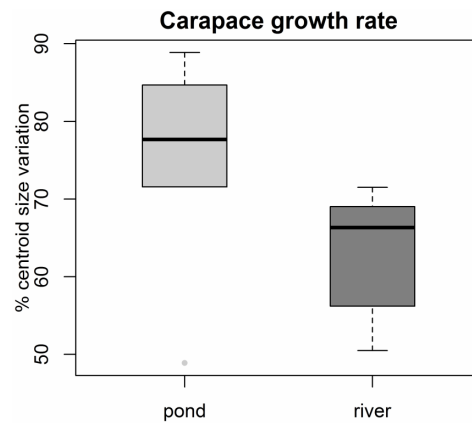


Figure 5. Carapace (centroid size) growth rate in "pond" vs "river" treated pond turtles

sexual dimorphism in different turtle species (Bonnet et al. 2010). However, in our experiment on controlled putative males, treatments produced significant effects only on the carapace, not on the plastron. If on the one hand plastron grew and enlarged allometrically as a time function, on the other hand carapace was the shell part more sensitive to environmental factors and subject to change more rapidly as a response to external stimuli (Rivera 2008). For instance, hydrodynamic conditions represent a widely recognised source of shape variation in several aquatic organisms (barnacles: Marchinko 2003; rainbowfish: McGuigan et al. 2003; intertidal algae: Boller & Carrington 2006), and habitat dependent dimorphism has

been found to be a heritable feature in rainbowfish (McGuigan et al. 2003). The absence of variation in plastron shape may be due to the time span we considered, perhaps too short to monitor even subtle changes recorded on the adults (Zuffi et al. 2007, Rivera 2008, Selman 2012), and that may occur during the whole life cycle (Congdon et al. 2003). The few available data on shell variation in different habitats suggest that upstream turtles are on average smaller than lower stream ones (Selman 2012), supporting previous hypotheses that habitat features may actually shape the overall morphology of freshwater turtles (Zuffi et al. 2007). Finally, in several unrelated organisms, there are examples showing that structures diverging in shape and growth rate represent adaptations to calm vs running waters (mudsnails: Holomuzki & Biggs 2006), but also to variable environments (sea turtles: Bjorndahl et al. 2003). In plants, a velocity gradient shaped several morphological features, resulting in a sharp decrease of all but one size-dependent traits (Puijalón & Bornette 2004). In vertebrates, a direct relationship between stream speed and organism morphology has been recently found in juvenile salmonids (Pakkasmaa & Piironen 2001, McGuigan et al. 2003).

We have interpreted our experimental running water habitat as a possible stressor which may induce growth differences. In a natural, environmental scenario of contrasting habitats, significantly different morphology in adult females on both carapace and plastron relative size were found (Zuffi et al. 2007, Selman 2012), as also recently pointed out by Germano & Riedle (2015). In turtles, unfortunately, there is no comparative information on growth patterns of juveniles living in contrasting habitats (natural or experimental). Thus, we can only reasonably speculate on the possible functions of shell shape fluctuations and variations comparing other organisms and their evolutionary traits. Variability of phenotypic patterns in running waters was recently proposed as a functional trade-off between the risk of flow-induced dislodgment and the risk of fish predation affecting shell armature frequencies of mudsnails (Holomuzki & Biggs 2006). It may be hazardous to predict the final size and form our experimental turtles could reach at maturity in a river system (Fritz 2003). However, the much wider, highly significant, variability of carapacial scutes of "river" treated turtles (see Fig. 4-5) denotes the occurrence of a phenotypic plasticity.

The overall shell plasticity we recorded sug-

gests that habitat conditions plays a key role in shaping turtles morphology at least during the earliest developmental and growing stages. Rivera et al. (2014), comparing slow vs fast-flow ecormorphs in three different Emydid species using a PT analysis, found a consistent variation of adult shell shape of fast-flow individuals when compared to individuals living in slow-flow regimes. Markedly stressing habitats, as already observed and pointed out by Selman (2012), produce a slow growth in carapacial size without affecting the overall shape. Our results are in line and concordant with those of Selman (2012), where upstream habitats have induced smaller size in resident adult individuals of Yellow-blotched Sawback. It remains to be tested (a matter virtually still unclear) if hatchlings coming from females living in running water habitats may display similar patterns to those we observed in pond habitats or do they follow different trajectories and patterns.

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