

ASSESSING THE MORPHOLOGICAL VARIABILITY OF *UNIO DELPHINUS* SPENGLER, 1783 (BIVALVIA: UNIONIDAE) USING GEOMETRIC MORPHOMETRY

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ABSTRACT

The morphological variability of freshwater bivalve species, observed between and within river basins, may hamper their correct identification, even by experienced researchers. Classic morphometric measurements, i.e. shell length, height and thickness, or their ratios, are generally insufficient to distinguish populations and/or species. These issues may be overcome using a geometric morphometric method, which allows analysis of the overall shape of the individual, independently of its size. Thus, we aimed to test the usefulness of two geometric morphometric tools, landmarks and sliding semilandmarks, to evaluate the morphological variability of *Unio delphinus* Spengler, 1783 in three habitats of the Guadiana Basin (SW Iberian Peninsula, Europe): estuary, river and stream. We used 13 landmarks located on the shell interior (at the teeth, muscle scars and pallial line) and 35 sliding semilandmarks for the shell contour. These morphometric analyses showed that the shell shape of *U. delphinus* differs significantly among different habitats. Estuarine and stream shells are the most disparate (James index = 649.114, permutation *P*-value < 0.001) and variability is not related to variations in shell size. The main differences in shell morphology are the following: (1) estuarine shells are more elongate, while riverine shells are more subovate; (2) the anterior curvature at the umbo is steeper in estuarine and riverine shells; (3) estuarine shells have an arched curvature at the ventral part of the shell, which is absent in specimens from the other habitats. Our data suggest that the morphology of *U. delphinus* shells might be influenced by the water flow characteristics of each habitat, since shells exhibited characteristics that are typically observed in freshwater mussels from lotic and lentic habitats.

INTRODUCTION

The phenotypic plasticity of freshwater mussel shells, observed between and within river basins (Ortmann, 1920; Baker *et al.*, 2004; Araujo, Gómez & Machordom, 2005; Watters, Hoggarth & Stansbery, 2009), hampers their correct identification even by experienced researchers and potentially compromises the effectiveness of monitoring and conservation programmes (Grigorovich, Angradi & Stepien, 2010; Shea *et al.*, 2011).

The morphological diversity that freshwater mussel species often exhibit and the use of the typological species concept (i.e. that species are defined by certain fixed characteristics) led to the description of thousands of species (Haas, 1940, 1969; Graf, 2011). Up to the mid-twentieth century, 4178 species had been described based on subtle differences in length, shape and colour; however, only 20% of those descriptions were considered valid by Haas (1940, 1969). Currently, the estimated diversity of freshwater mussels is about 870 species (Graf & Cummings, 2007). The clarification of which taxa constitute valid species is mainly due to advances in genetics (Graf & Cummings, 2007). Still, species identification cannot rely solely on genetics; there

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are genetic boundaries between taxa, but morphological aspects are also essential for routine identification.

In the case of the genus *Unio* Philippon in Retzius, 1788, although basic shell measures (length, height and width) aid in discriminating species, they are not fully conclusive, since the growth of *Unio* species is strongly affected by environmental conditions (e.g. Zieritz & Aldridge, 2009; Hornbach, Kurth & Hove, 2010; Zieritz *et al.*, 2010). However, recent research has led to a paradigm shift on how morphometric studies are perceived by researchers (Adams, Rohlf & Slice, 2004).

Morphometrics, which is the study of shape variation and covariation among variables (Bookstein, 1991, Dryden & Mardia, 1998), was traditionally restricted to a set of multivariate methods applied to several linear measures. More recently, geometric morphometric methods have enabled the partitioning of shape and size components, preserving the main geometric properties of the specimens, while generating a visual representation and determining shape variables that can be analysed statistically. Geometric morphometry is often described as a 'revolution' in the area of morphometrics (Adams *et al.*, 2004) and it has been successfully applied in many fields of biological research (e.g. ecology, evolution, ontogeny and taxonomy). Because of their hard and stable shells, bivalves are an excellent group for the application of geometric morphometric methods (Rufino *et al.*, 2006). Historically, geometric morphometric techniques were based on two main approaches: (1) contour/outline analysis, where the border of a specimen is analysed (Rohlf & Archie, 1984) and (2) landmark analysis, where the change in the position of homologous points identified in each specimen is quantified (Zelditch, Swiderski & Sheets, 2012). Both approaches provide different types of information and can be applied in distinct circumstances. Initially, geometric morphometric studies on bivalves used mainly contour-based methods (e.g. Ferson,

Rohlf & Koehn, 1985; Innes & Bates, 1999). There are several studies of Unionoida that have used Fourier analysis (Rohlf & Archie 1984; Scholz & Hartman, 2007b). Most recent studies have employed landmarks, a combination of both approaches (i.e. landmarks and contours) or sliding semilandmarks, a newer technique used to analyse curves (e.g. Perez, Bernal & Gonzalez, 2006). These techniques have been successfully applied to distinguish between similar species of bivalves (Rufino *et al.*, 2006; Costa *et al.*, 2008), between wild and aquaculture stocks (Valladares, Manríquez & Suárez-ISLA, 2010) and between fossil and modern taxa (Aguirre, Perez & Sirch, 2006), and also to detect ontogenetic shape changes (Márquez *et al.*, 2010) and to analyse geographic variation in shape (reviewed by Rufino *et al.*, 2012).

Bivalve shell morphology is linked to a series of endogenous (genetic and physiological) and exogenous factors (biotic and abiotic interactions) (e.g. Scholz & Hartman, 2007a, b; Van Bocxlaer & Van Damme, 2009; Zieritz & Aldridge, 2009). Indeed, establishing relationships between bivalve morphology and habitat characteristics is crucial for conservation efforts. This is so, because a bivalve's survival might be critically compromised during habitat modifications or when specimens are translocated for repopulation purposes (Hornbach *et al.*, 2010).

In the Guadiana River Basin (hereafter, Guadiana Basin) (SW Iberian Peninsula; Fig. 1), two *Unio* species are present: *U. delphinus* Spengler, 1783 and *U. tumidiformis* Castro, 1885 (Reis, 2006; Araujo *et al.*, 2009; Reis & Araujo, 2009; Reis, Machordom & Araujo, in press). These species correspond to the traditional Iberian taxa previously identified as *U. pictorum* L., 1758 (*U. delphinus*) and *U. crassus* Philippon in Retzius, 1788 (*U. tumidiformis*), having recently been recognized as their respective sister species based on molecular, morphological, reproductive and ecological data (Araujo *et al.*, 2009; Reis & Araujo, 2009). *Unio delphinus* is the most common freshwater

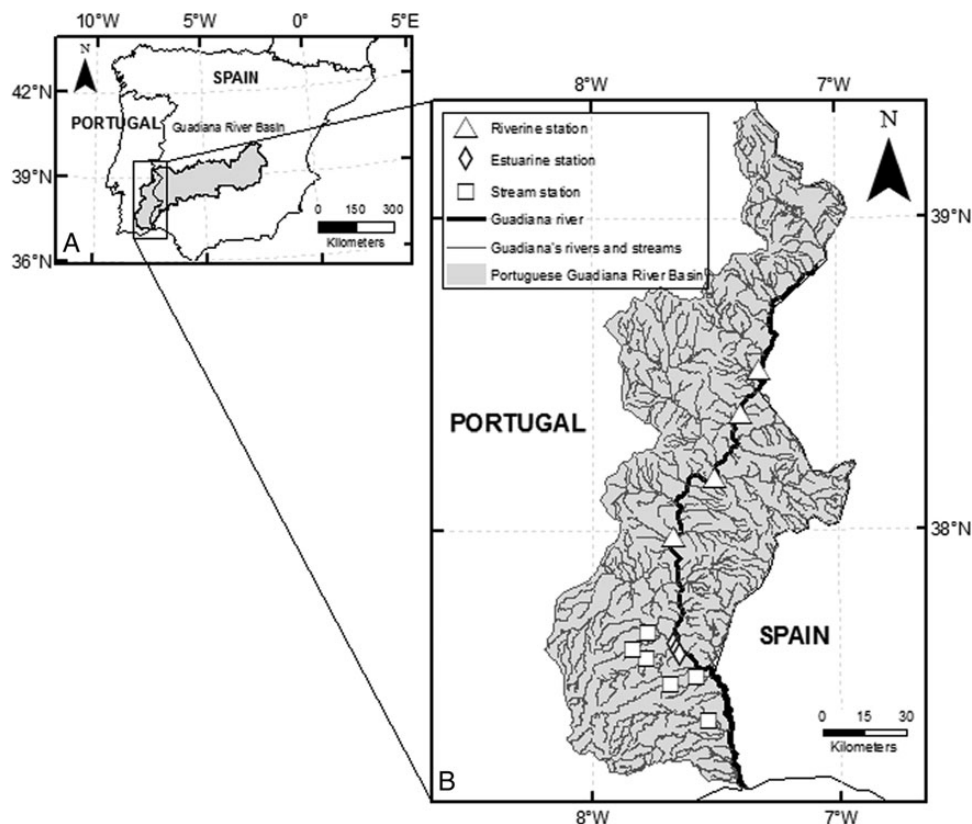


Figure 1. Location of the Guadiana Basin (A) and of the sampling stations located along the Portuguese Guadiana Basin (B).

mussel in the Guadiana Basin. Its distribution includes all major Atlantic Iberian basins, as well as Moroccan Atlantic basins (Araujo *et al.*, 2009; Morais *et al.*, 2009; Reis *et al.*, in press). *Unio delphinus* inhabits a wide variety of habitats and ecological conditions, including lakes to rivers, ranging from oligotrophic waters to semiarid streams (Reis, 2006; Araujo *et al.*, 2009). On the other hand, *U. tumidiformis* is restricted to the southwest of the Iberian Peninsula and is usually one of the least abundant species found (Reis & Araujo, 2009; Reis *et al.*, in press). Most of the currently known populations are located in the Guadiana Basin, where they inhabit medium-sized, Mediterranean-type, intermittent streams (Reis & Araujo, 2009). Distinguishing between the two species is difficult, given their morphological resemblance (Reis, 2006; Araujo *et al.*, 2009; Reis & Araujo, 2009; Reis *et al.*, in press). Reis & Araujo (2009) and Reis *et al.* (in press) found that the shell width/length ratio is useful but not 100% reliable for distinguishing them. Key morphological differences such as the umbo sculpture or the hinge are often not visible in live specimens or not preserved due to erosion (Reis & Araujo, 2009). The morphological differences between the two species can only be evaluated after analysing their intraspecific morphological variation.

Our objective was to assess the morphological variability of *U. delphinus* in three different habitats of the Portuguese Guadiana Basin (estuary, river and intermittent streams). Our methodological approach allowed us to test the usefulness of combining two geometric morphometric tools (landmarks and sliding semilandmarks) to evaluate the morphological variability of freshwater mussels.

MATERIAL AND METHODS

Characterization of the Guadiana Basin

The Guadiana Basin is the fourth largest in the Iberian Peninsula (Fig. 1). The Guadiana originates in Lagunas de Ruidera (Spain) and drains into the Atlantic Ocean near the city of Vila Real de Santo António (Portugal). The river has a total length of 810 km and its basin has an area of 66,889 km² (Fig. 1). The river flow varies among and within years. The average annual river flow oscillates between 8 and 63 m³ s⁻¹ during dry years, 170–190 m³ s⁻¹ in average years and 412–463 m³ s⁻¹ in wet years (Bettencourt *et al.*, 2003). Due to the Mediterranean climate of the area, extensive stretches of the basin's streams dry out leaving a series of isolated pools, where the aquatic fauna has to cope with severe environmental conditions (e.g. high temperature and low oxygen concentration) in the smallest pools. The river's mesotidal estuary occupies an area of 22 km². Its average depth is 6.5 m and the tidal limit is located 70 km upstream the river mouth (Bettencourt *et al.*, 2003).

The sustainability of the Guadiana Basin ecosystem is threatened by three main factors (Morais, 2008): (1) aquifer overexploitation in the upper basin, which supports extensive irrigation areas; (2) nonpoint-source pollution originating in these irrigation areas from industry, decommissioned mines, untreated sewage, sewage treatment plants and landfill and (3) intense damming of the basin with a total of 12,730 hm³ of water stored in reservoirs, retaining about 150% of the average annual rainfall (UNEP, 2006). For further details on the impacts caused by damming in the Guadiana Basin, see Morais (2008).

Sample processing and data analyses

Shells of *U. delphinus* were collected in three distinct habitats of the Guadiana Basin: estuary ($n = 3$ locations), river ($n = 4$ locations) and from isolated pools in intermittent streams ($n = 6$

locations) (Fig. 1B). Specimens were collected by hand in the river and streams and with a clam dredge in the estuary. Specimens were identified by three researchers and only specimens with concordant identifications were considered for the analyses. Thus, 61 individuals were used for morphometric analyses: 8 specimens from the estuary, 26 from the river and 27 from the intermittent streams (isolated pools). Only undamaged valves from dead animals were analysed. A digital image of the inner face of the left valve of each specimen was taken in a posterior-anterior orientation. On each image, 13 landmark points located on the shell interior and 35 sliding-landmarks on the shell outline were marked with tpsDig to quantify shape (Fig. 2) (Rohlf, 2010). The chosen landmarks were unambiguously visible in all specimens from the different habitats. The number of sliding semilandmarks was determined by visual inspection, using the criterion of the minimum number of points needed to properly define shape complexity. Landmarks and sliding semilandmarks positions were rotated, scaled (to unit centroid size) and translated through Generalized Procrustes Analysis. (For more details on geometric morphometric methodologies using landmarks, see Bookstein, 1991; Dryden & Mardia, 1998; Adams *et al.*, 2004; Zelditch *et al.*, 2012.) Sliding semilandmarks were obtained through curve-tracing methods and were permitted to slide along the axis parallel to the line between two adjacent sliding semilandmarks until their spacing minimally impacted the amount of shape difference between forms. The final coordinates of the configuration obtained were incorporated into subsequent analyses.

Multivariate Procrustes permutation analysis of covariance (ANCOVA) was used to test for differences among shell shapes of the three habitats (estuary, river and stream), using all shape variables (landmarks and sliding semilandmarks) and in relation to centroid size. Relative warp (RW) analysis using all shape variables (similar to principal component analysis) was used to provide an ordination of all the specimens in a morphological space. Differences between mean shell shapes of each group were tested by the James index, which tests for differences

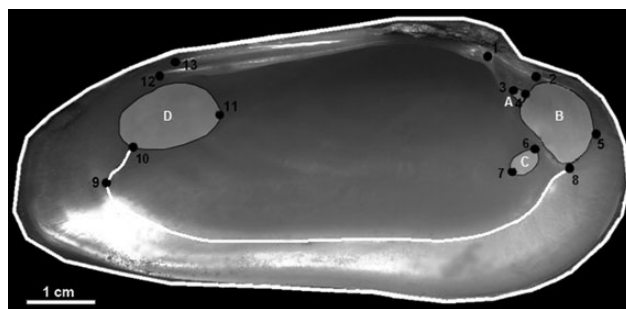


Figure 2. Left valve of *Unio delphinus* with the location of the landmarks (black dots, numbered from 1 to 13) and sliding semilandmarks (white contour line; the sliding semilandmarks were positioned along this line) used to evaluate the morphological variability of the shells collected in three habitats (streams, river and estuary) of the Portuguese Guadiana Basin. Legend: white line, pallial line scar; A, anterior retractor muscle scar; B, anterior adductor muscle scar; C, protractor muscle scar; D, posterior adductor muscle scar; 1, posterior insertion point of the pseudocardinal tooth; 2, anterior insertion point of the pseudocardinal tooth; 3, upper point of the anterior retractor muscle; 4, lower point of the anterior retractor muscle; 5, anterior point of the anterior adductor muscle; 6, anterior point of the protractor muscle; 7, posterior point of the protractor muscle; 8, confluence point between the pallial line and the anterior adductor muscle; 9, posterior point of the pallial line; 10, confluence point between the pallial line and the posterior adductor muscle; 11, anterior point of the posterior adductor muscle; 12, insertion point of the lower lateral tooth; 13, insertion point of the upper lateral tooth.

between two sample populations using tangent coordinates, with the respective significance being calculated through Bootstrap techniques (permutation procedures) (Amaral, Dryden & Wood, 2007). Distances between groups were inferred using Riemannian shape distance (Dryden & Mardia, 1998). All statistical analyses and graphical representations were performed with the software R (R Development Core Team, 2010) using the packages ‘Vegan’, ‘Morpho’, ‘shapes’ and ‘geomorph’ (Adams & Otárola-Castillo, 2012; Dryden, 2012; Oksanen *et al.*, 2012; Schlager, 2012).

RESULTS

The shell shapes of *Unio delphinus* living in the estuary, river and streams are significantly different from each other, as are shape differences during growth (permutation multivariate ANCOVA Centroid size MS = 0.014, $P < 0.001$; habitat MS = 0.009, $P < 0.001$ and interaction MS = 0.003, $P = 0.056$). The shapes of river shells are significantly different from estuary shells (James index = 649.114, permutation P -value < 0.001) and stream shells (James index = 4887.138, permutation P -value < 0.001). Stream and estuary shells also differ significantly (James index = 2233.453, permutation P -value < 0.001). The mean shape configuration of *U. delphinus* in each habitat shows that river and estuary shells have a more pronounced umbo than stream shells. Estuary shells are slightly more elongated than the others. Riemannian shape distance shows that river shells are closer to stream individuals ($\rho = 0.039$), followed by river *vs* estuary ($\rho = 0.049$), whereas the most different ones are estuary *vs* stream specimens ($\rho = 0.052$) (Fig. 3).

The RW analysis confirms the mean shape analysis results, since the three groups are distinguishable using the first three RWs (Fig. 4). RW1 *vs* RW2 separate stream shells from river and estuary specimens (Fig. 4A), whereas RW2 *vs* RW3 differentiates samples from the estuary, river and streams (Fig. 4C). Generally, the first axis (RW1) relates to shape changes associated with size (i.e. allometry), even when the size component has been removed. RW2 seems to distinguish estuary + river specimens from stream specimens. RW3 shows a shape change from river, to stream and estuarine animals (Fig. 4C).

DISCUSSION

The combined use of two geometric morphometric tools, landmarks and sliding semilandmarks, revealed the subtle morphological differences exhibited by *Unio delphinus* among habitats within the same hydrographic basin. These subtle differences are difficult to describe based on simple comparative observations of specimens from different habitats. However, mean-shape

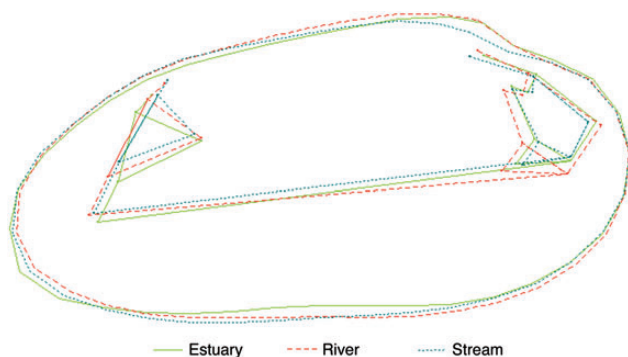


Figure 3. Mean shape of *Unio delphinus* collected in three habitats of the Portuguese Guadiana Basin (estuary, river and stream) after generalized Procrustes superimposition analysis.

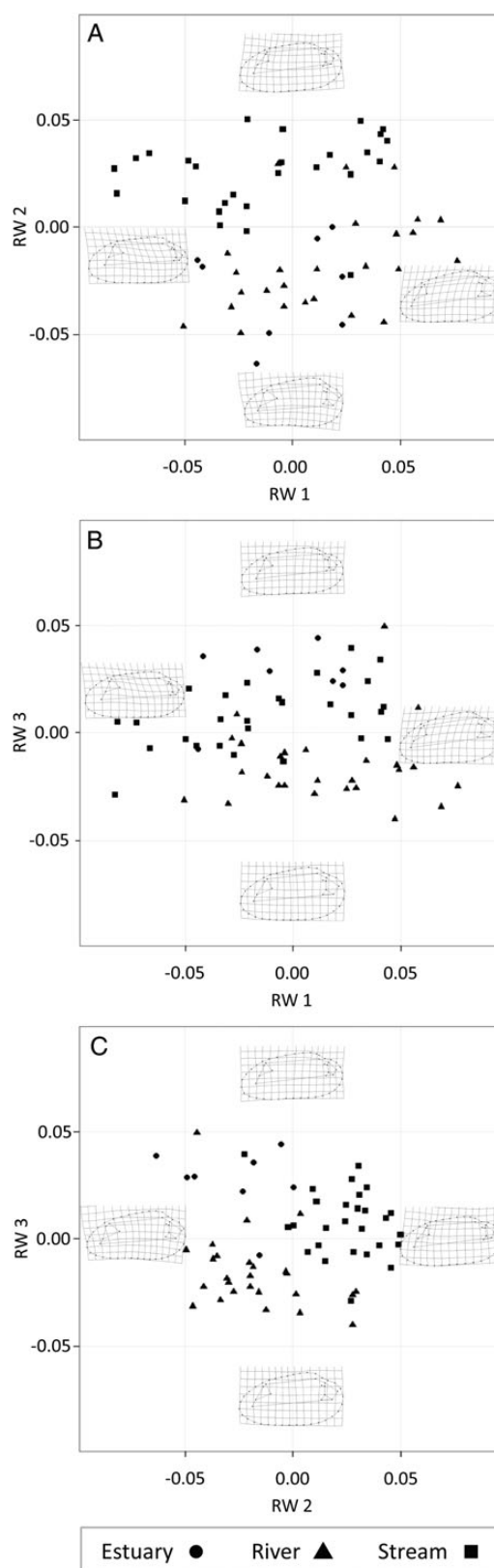


Figure 4. Relative warp analysis biplots (A, RW1 *vs* RW2; B, RW1 *vs* RW3; C, RW2 *vs* RW3) calculated on *Unio delphinus* landmarks and sliding semilandmarks of specimens collected in three habitats of the Portuguese Guadiana Basin (estuary, river and stream). The variance explained by each RW was as follows: RW1: 36.5%; RW2: 23.0%; RW3: 12.4%. Synthetic shell outlines of ‘extreme’ morphotypes are displayed with the anterior margin facing to the right and the dorsal margin to the top of the figure.

statistical analysis allowed us to summarize these differences. In the Guadiana Basin, estuary specimens are more elongate than river and stream specimens, while river specimens are more subovate than specimens from the other habitats (Fig. 3). There is a gradient of shapes, from slender to more subovate, from estuary to streams to river. Other obvious differences are the anterior curvature at the umbo, which is steeper in estuary and river specimens, and the arched ventral curvature of estuary specimens which is not present in specimens from the other two habitats (Fig. 3).

As demonstrated in previous studies dealing with the intraspecific phenotypic plasticity of molluscan shells, the morphological variability of *U. delphinus* might depend on several abiotic and biotic factors (e.g. Trussell, 2002; Laudien, Brey & Arntz, 2003; Pascoal *et al.*, 2012), such as water velocity (Trussell, 2002), wave exposure (Boulding, Holst & Pilon, 1999), water depth (Claxton *et al.*, 1998), sediment grain size (Hornbach *et al.*, 2010), sex (Zieritz & Aldridge, 2011 and references therein), infestation by parasites (Zieritz & Aldridge, 2011), abundance of predators (Edgell & Neufeld, 2008; Neo & Todd, 2011; Bourdeau, 2012) and food availability (Preston & Roberts, 2007).

Morphological differences might also result from genetic differences between populations; however, ecophenotypic differences might exist even when there is no genetic variation (Bagatini *et al.*, 2005; Sousa *et al.*, 2007; Carstensen *et al.*, 2009; Rabaoui *et al.*, 2011). The lack of significant genetic differentiation of *U. delphinus* along the Guadiana Basin suggests that phenotypic plasticity is influenced by some of the mentioned biotic and abiotic factors (Reis *et al.*, in press). However, factors like wave exposure and sex (Iberian *Unio* spp. show no sexual dimorphism) are not applicable (Araujo *et al.*, 2009). Other factors are unlikely to have a significant effect because they are relatively uniform, and their prevalence is low along the basin (e.g. infestation of parasites and abundance of predators) (J.R., personal observations). Other factors, like calcium concentration, are sufficiently consistent across all habitats so as not to cause a differentiating effect among the shells (P.M., unpubl.). Among all possible factors, water velocity is widely cited as the most influential in determining the morphology of Unionidae (e.g. Ortmann, 1920; Watters, 1994; Zieritz & Aldridge, 2009; Hornbach *et al.*, 2010).

Different ecomorphs reveal their adaptability to lotic and lentic habitats, because certain shell shapes offer enhanced burrowing capacity to cope with hydrological variability (Ortmann, 1920; Zieritz & Aldridge, 2009). Usually, specimens from lotic habitats have more elongated and arched shells than specimens from lentic habitats, allowing lotic individuals to reburrow readily after dislodgment (Baker *et al.*, 2004; Hornbach *et al.*, 2010). For example, in the Guadiana Basin, the steeper umbonal curvature of river and estuary specimens might assist in burrowing (Watters, 1994; Hornbach *et al.*, 2010). The ventral arching observed in estuary specimens might be an adaptation to tidal currents, because it probably provides additional anchoring to cope with water flowing in two opposite directions. The dorsal arching, or the more subovate form of river and stream specimens, enables the foot to extend further into the substrate and increase anchoring and stability under turbulent water conditions (Zieritz & Aldridge, 2009).

The morphological adaptations of *U. delphinus* observed in the Guadiana Basin seem to match the general hydrological characteristics of the system. The estuary is characterized as a permanent lotic habitat due to its tidal currents, with laminar flow characteristics. Conversely, Guadiana's streams are characterized as lentic habitats during most of the year, due to the isolated pools that exist; however, streams have turbulent flow characteristics during periods of higher inflow. The Guadiana River has lotic habitats with laminar and turbulent flow characteristics, and also temporary lentic microhabitats formed along the banks

during periods of very low inflow (INAG, 2012). Still, it is plausible that the estuary and the intermittent streams are more unstable environments than the river itself, due to their dual directional flow and extreme flow variation, respectively. Indeed, freshwater mussels prefer stable or hydraulically sheltered habitats (Allen & Vaughn, 2009; Negishi *et al.*, 2011) and often these areas are microhabitats created by rocks and roots, and it is common to find specimens burrowed horizontally at the banks between tree roots (Araujo *et al.*, 2009). This is a general phenomenon and may have little influence on shell morphology among habitats.

We recommend that if the translocation of specimens is a viable option to repopulate certain Guadiana streams, then conservation managers should use specimens from other stream habitats with viable populations, rather than river or estuary specimens. This strategy should ensure that the mussels will adapt physiologically, particularly to the extreme abiotic conditions that occur in summer. The morphological characteristics of these mussels will also be more suited to cope with the flow characteristics, as highlighted by Hornbach *et al.* (2010). The use of river specimens for translocation to streams should not be excluded since they are morphologically more similar to stream specimens than are those from the estuary. However, a translocation programme can only be effective if managers tackle the existing threats to *U. delphinus* in Guadiana streams: firstly, the presence of nonindigenous fish species, which may compromise their reproduction success and, secondly, water abstraction from the isolated pools for irrigation, which can destroy a significant fraction of a population (Collares-Pereira *et al.*, 2000; Barea-Azcón *et al.*, 2009).

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