



Clinal variation in carapace shape in the South American freshwater crab, *Aegla uruguayana* (Anomura: Aeglidae)

FEDERICO GIRI^{1,2} and PABLO COLLINS^{1,3*}

¹*Instituto Nacional de Limnología (CONICET-UNL), Pje El Pozo s/n, CP3000, Santa Fe, Argentina*

²*Facultad de Humanidades y Ciencias, Universidad Nacional del Litoral, Pje El Pozo s/n, CP3000, Santa Fe, Argentina*

³*Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Pje El Pozo s/n, CP3000, Santa Fe, Argentina*

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South America has been influenced by different geoclimatic events ever since its separation from Africa. The inland water fauna has evolved in response to the changing landscape. Currently, there are indications of variations in populations, occurring to different degrees that would indicate a clinal pattern in morphology. Among South America's fauna, the freshwater anomuran, *Aegla*, is an enigmatic group as a result of its endemism and is composed of only one genus. Of all the species in this family, *Aegla uruguayana* has the broadest distribution. Its native habitats have been influenced by several marine transgressions during the Miocene–Quaternary Periods; thus, it is likely that their current distribution has been more recent. Its habitat spreads across a number of isolated basins and sub-basins that display distinct degrees of isolation/connection, making clinal variation patterns in the morphology of this species possible. The present study aimed to evaluate the pattern of carapace shape variation in *A. uruguayana* and how it relates to the isolation and/or connection of populations from different basins and sub-basins, allowing the determination of any extant clinal patterns. The specimens studied belong to 25 separate populations, representing all areas in which the species currently exists. A total of 523 crabs were analyzed. We identified 13 landmarks and four semi-landmarks in the carapace. The aeglids were divided into seven size intervals to avoid an allometry effect. In each size category, shape relationships analyzed by principal component analysis suggest a geographical pattern corresponding to the distribution of the populations studied. An evaluation of covariation between body shape and geographical coordinates reveals a strong pattern and shows that population distribution had a significant effect on species morphology. Additionally, according to covariance analysis, the variation in shape was not associated with the environmental variables studied. We observed a clinal pattern throughout the species distribution, which could be attributed to genetic drift. It is possible that this process is being amplified by the geographical isolation of the basins, differences in environmental characteristics, and low dispersal ability. © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2014, **113**, 914–930.

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INTRODUCTION

'Earth and life evolve together' (Croizat, 1964). Different macro-events have occurred throughout the earth's history that provoked changes in the habitats

it provided and, in turn, affected plant and animal populations. In the case of the freshwater environments of southern South America, events such as the Gondwana separation, Andes uplift, marine transgressions, and glaciations have influenced the landscapes and the abiotic characteristics of the environment, which have then affected the distribution of the biota over various time scales (Suguio

*Corresponding author. E-mail: pagcollins@gmail.com; pcollins@inali.unl.edu.ar

et al., 1985; Lundberg *et al.*, 1998; Ribeiro, 2006; Collins, Giri & Williner, 2011; Anger, 2013). Furthermore, the history of hydrographic basins on this continent is marked by changes in the direction of the rivers, as well as by changes in both flow rate and abiotic conditions. Some modifications were sufficiently extreme to provoke separation and isolation between species populations (Ribeiro, 2006).

Among decapods, Aeglidae represent the only family of anomurans inhabiting southern South American freshwater environments. The sole extant genus, *Aegla* (Leach, 1820), includes approximately 72 species, and new species are currently being described (Bond-Buckup *et al.*, 2008; 2010; Santos *et al.*, 2012). Three species are broadly distributed, whereas the remaining species are endemics (Pérez-Losada *et al.*, 2004; Bond-Buckup *et al.*, 2010). *Aegla uruguayana* (Schmitt, 1942) is one of the species with a broad distribution, occurring in very heterogeneous environments (large and small rivers, mountain streams, ponds, lagoons, and lakes) (Bond-Buckup & Buckup, 1994; Giri *et al.*, 2014). Some populations within this species are isolated from one another, occurring in different basins. Moreover, an important characteristic of the genus is its limited ability for movement and migration (López, 1965; Xu *et al.*, 2009). In particular, molecular studies of *A. uruguayana* have shown that the dispersal ability of this species is lower than that required to counteract genetic differentiation as a result of drift (Giri *et al.*, 2014).

The regions in which *A. uruguayana* occurs are the La Plata Basin (e.g. Paraná and Uruguay Rivers), isolated small rivers and streams on the Atlantic Ocean slope (e.g. Maldonado Stream), and the Lagoa dos Patos Basin (e.g. dos Patos, and Mirim Lagoons) (Fig. 1). This area was influenced by several marine transgressions during the Miocene–Quaternary Periods (Potter, 1997; Lundberg *et al.*, 1998), which may have provoked the move and/or separation of the initial populations. As noted previously, certain populations of *A. uruguayana* are currently found in environments that are not interconnected (Bond-Buckup & Buckup, 1994). However, other populations have either ephemeral or stable connections that may allow movement and population integration (i.e. different degrees of gene flow) (Giri *et al.*, 2014). Such gene flow could lead to the manifestation of different phenotypes, which could be observed by studying the carapace shape of individuals from different populations.

Current knowledge regarding *A. uruguayana* has been provided by research of biometric characteristics of the genus (Vaz-Ferreira, Gray & Vaz-Ferreira, 1945; Ringuelet, 1948, 1949; Bond-Buckup & Buckup, 1994); moreover, molecular information on the genus

has also been characterized (D'Amato & Corach, 1997a, b; Pérez-Losada *et al.*, 2004; Bitencourt, 2007; Bartholomei-Santos, Roratto & Santos, 2011; Giri *et al.*, 2014). Information about the shape of the carapace has been found to aid in the differentiation of males from females (Giri & Collins, 2004) and the relationship between the shape of the coxa and the dorsoventral shape of the fifth pereopod has been used to identify whether gonadal maturation has occurred (Collins, Giri & Williner, 2008). However, the morphological variability between distinct populations of *A. uruguayana* throughout its distribution has never been evaluated. Populations that are more distant (different sub-basin) have different genetic conditions compared to those that live in nearer site, justified by a low migration rate among populations (Giri *et al.*, 2014).

Morphological variations have been recognized as indicators of clinal patterns in different species, developing along a temporal scale or geographical area as a result of population differentiation accompanied by divergent phenotypic expressions that are the manifestation of the differentiation in the genotypic (Cardini, Anna-Ulla & Elton, 2007; Vonlanthen *et al.*, 2009). This variation can be product of the level of interchange of individuals between populations more or less distant, with barriers or facilitators of movement. Also, plastic responses in morphology as a result of environmental conditions could be a response added to the clinal variation (Miner *et al.*, 2005). Unfortunately, such studies pertaining to decapods are scarce. Among them, Gotelli, Gilchrist & Abele (1985) found a morphological clinal variation in the crab *Teleophrys cristulipes* (Stimpson, 1860). Additional patterns of variation in decapods, relating to environmental differences present in distinct hydrographic fronts, have been found in portunoid crabs from the Mediterranean (Almeria/Oran) where the difference in abiotic conditions is a structural factor in the phenotype expression (Rufino, Abelló & Jones, 2004). Moreover, other studies have reported patterns associated with decapod gene flow and dispersal in isolated and non-isolated populations, by promoting or maintaining the divergence between populations or species through the same barrier (Stewart, 1997; Daniels, Stewart & Gibbons, 1998; Hewitt, 1999; Daniels, Stewart & Burmeister, 2001; Irwin, Irwin & Price, 2001; Daniels, Stewart & Cook, 2002; Daniels, 2003; Pfeiler *et al.*, 2005; Trontelj, Machinob & Sketa, 2005; Daniels, Gouws & Crandall, 2006; Schubart & Huber, 2006; Pedraza-Lara *et al.*, 2010; Stefani *et al.*, 2011; Scalici & Bravi, 2012; Giri *et al.*, 2014).

It is known that the basins in which *A. uruguayana* can be found are isolated from one another and that, within each basin, there are rivers with varying

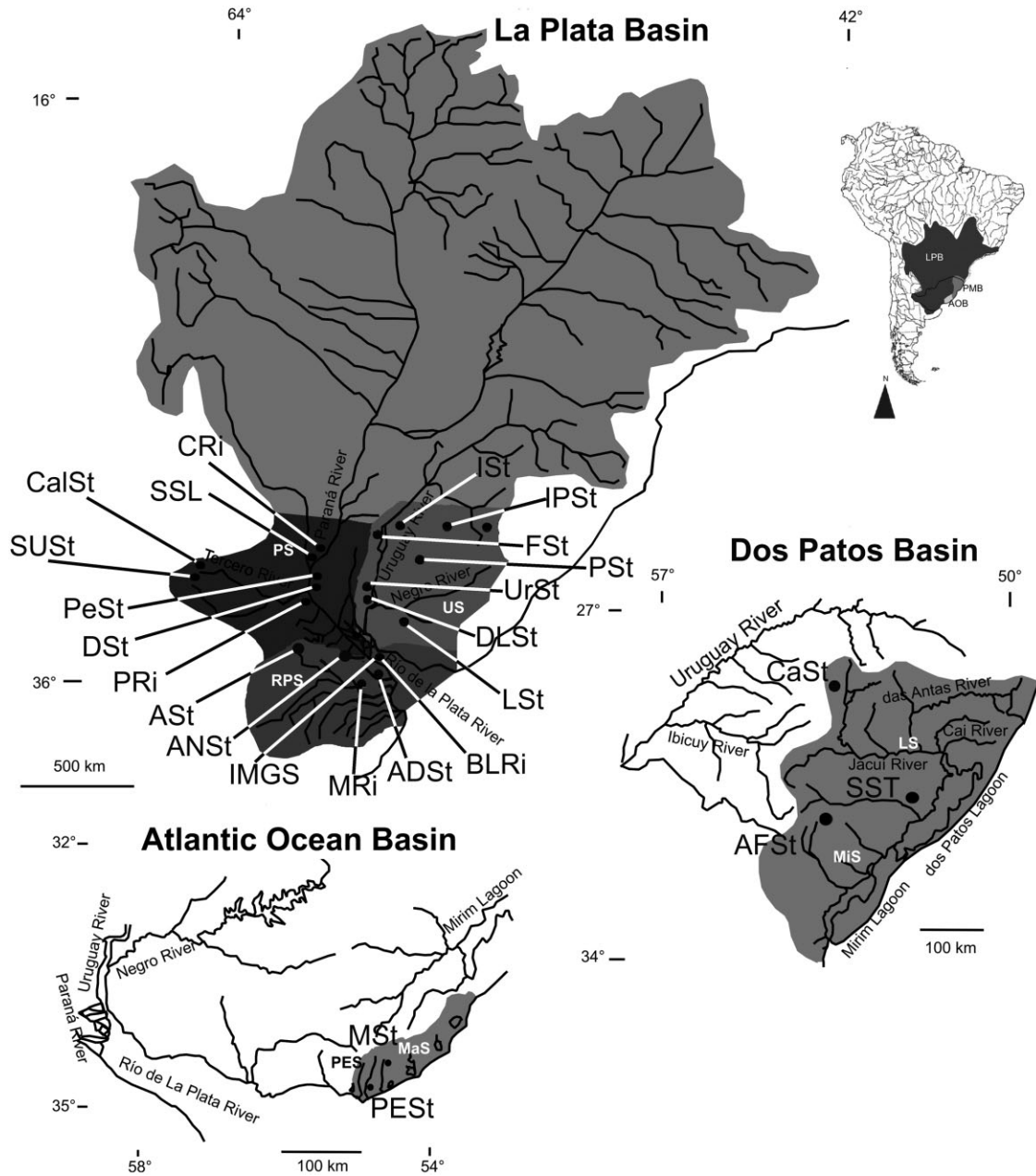


Figure 1. Basins of southern South America where *Aegla uruguayana* live, indicating their sub-basins, and locations of sampled environments. AOB, Atlantic Ocean Basin; LPB, La Plata Basin; LPS, dos Patos Sub-basin; MaS, Maldonado Sub-basin; MiS, Mirim Sub-basin; PES, Punta del Este Sub-basin; PMB, Lagoa dos Patos Basin; PS, Paraná Sub-basin; RPS, Río de la Plata Sub-basin; US, Uruguay Sub-basin. 1, Segunda Usina Stream (SUST); 2, Calamuchita Stream (CalSt); 3, Setúbal Shallow lake (SSL); 4, Colastiné River (CRI); 5, Las Pencas Stream (PeSt); 6, Doll Stream (DSt); 7, Paraná River (PRi); 8, Areco Stream (ASt); 9, Streams in Martin Garcia Island (IMGS); 10, Anchorena Stream (ANSt); 11, Adrogué Stream (ADSt); 12, Matanza River (MRi); 13, Brazo Largo River (BLRi); 14, De las Leches Stream (DLSt); 15, Urquiza Stream (UrSt); 16, Federación Stream (FSt); 17, Ibaá Stream (ISt); 18, Ibirapuita Stream (IPSt); 19, Palacio Stream (PSt); 20, Lunarejo Stream (LSt); 21, Candiota Stream (CaSt); 22, Amaral Ferrador Stream (AFSt); 23, Seival Stream (SSt); 24, Maldonado Stream (MSt); 25, Punta del Este Stream (PESt).

degrees of connectivity. Furthermore, *A. uruguayana* exist in this environment with a very limited capacity for movement and it can be deduced that geoclimatic processes have moulded this environment on a relatively recent time scale. Given these facts, we hypothesize that the carapace shape may vary between different populations of this species and these variations may have manifested into a clinal pattern. The present study aimed to analyze the pattern of shape variation in *A. uruguayana* as it relates to the degree of connectivity and/or isolation of populations throughout its distribution, potentially showing evidence of a clinal pattern in morphology that has developed across the geographical distribution of this species through the force provoked by geoclimatic events in the time.

MATERIAL AND METHODS

ANIMAL PROVENANCE AND STUDY AREA

Selected populations of *A. uruguayana* represent the entire spatial distribution of this species (Fig. 1). The specimens studied are from 25 distinct populations. In total, 523 aeglids (259 males and 264 females) were analyzed (Table 1). Animals were collected with handnets from their natural environments and obtained from museums, such as the Museo Argentino de Ciencias Naturales Bernardino Rivadavia (MACNBR) (Argentina), Museo de Ciencias Naturales de La Plata (MCNLP) (Argentina), Museo de Ciencias Naturales Florentino Ameghino (MCNFA) (Argentina), the collection of Fundación Miguel Lillo (CFML) (Argentina), and the collection of Universidade Federal do Rio Grande do Sul (UFRGS) (Brazil). Additionally, aeglids were donated by the Laboratorio de Fisiología Animal Comparada, Departamento de Biodiversidad y Biología Experimental de la Facultad de Ciencia Exactas y Naturales de la UBA (LFAC-UBA) (Argentina). Animals obtained, from either the environment or a museum, were in the intermoult stage of the moult cycle and with the carapace in optimal condition. The animals are currently being preserved in a solution of 70–80% alcohol.

Environmental variables such as pH, temperature, dissolved oxygen, conductivity, and water transparency were recorded by digital sensors (HANNA model HI9143; HI98130), and substrate type was measured at the sampling sites for 16 of the populations (see Appendix, Table A1).

DATA ACQUISITION

The dorsal carapace of each aeglid was photographed with a Sony DSC-F717 digital camera (5.1 MP). We identified 32 landmarks on the carapace to use as a baseline configuration. These landmarks were

reduced to 13 landmarks and four semi-landmarks (Fig. 2) upon completion of a symmetrization process (Giri & Loy, 2008) because the carapace of *A. uruguayana* was considered to be represented by a symmetrical object, as in Mardia, Bookstein & Moreton (2000). This process relies on the superposition of the right and left halves of the carapace, as defined by the symmetry axis (i.e. landmarks 1 and 17), following the definition of the right and left consensus configurations of each crab. The semi-landmarks were defined by using the Procrustes superimposition procedure (Rohlf & Slice, 1990). The minimum bending energy criterion was used for fixation (Bookstein *et al.*, 2002; Pérez, Bernal & González, 2006). The superimposition method used was the generalized Procrustes analysis method (Rohlf & Slice, 1990). To compare only the shape of the objects, position and orientation were removed by translation and rotation, whereas the effect of size was removed by proportionalization with TPSRELW (Rohlf, 2009).

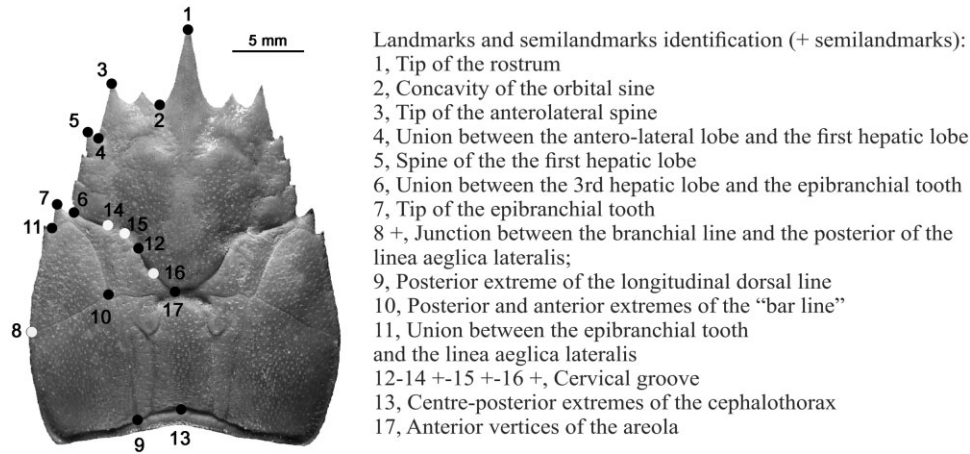
SEXUAL DIMORPHISM AND ALLOMETRY

Sexual dimorphism was evaluated in each population of *A. uruguayana* with by multivariate analysis of variance (Hotelling's test). Relative warps (RWs), which explained up to 70% of the variation as a result of sexual dimorphism, were used. However, the effect of sexual dimorphism on the data was minimized in the present study because the posterior vertex of carapace was not considered, which is strongly influenced by sexual dimorphism (Hepp *et al.*, 2012; Trevisan *et al.*, 2012), in accordance with the methods used by Giri & Collins (2004) and Giri & Loy (2008).

Ontogenetic allometry was evaluated in each population (if $N \geq 10$) by using a regression of shape variability on the natural log centroid size (lnCS) using a Goodall's *F*-test (Monteiro, 1999; Klingenberg, Barluenga & Meyer, 2003). A permutation with 1000 iterations was run to simulate the null hypothesis, which assumed the independence of size and shape (TPSREG; Rohlf, 2011). To avoid the influence of size (i.e. to evaluate the allometric relationship), crabs were divided into size categories determined by carapace length (CL) (seven groups, see Appendix, Table A2). First, we defined the size of an individual at sexual maturity (1.15 cm), which allowed specimens to be classified as juveniles (r_0) (< 1.15 cm of CL) or adults (> 1.15 cm of CL) (Viau *et al.*, 2006). Second, with regard to adults, the interval ranges (i.e. r_1 to r_6) were selected based upon the difference between mean moult increase (1.632 cm) of carapace length and their standard deviation (± 1.196 cm) corresponding to 0.43 cm. This criterion was selected by considering the variation of moult increase among the

Table 1. Studied populations of *Aegla uruguayana* identified by basin, sub-basin, code identification of each population, geographical coordinate, number of aeglids analyzed (*N*), number of males and females, and carapace size of individual in each analyzed population (mean ± SD)

Basin	Sub-basin	Population and code identification	Geographical coordinate	<i>N</i>	Male/female	Carapace size (cm)
La Plata (LPB)	Paraná (PS)	1 – Segunda Usina (SUSt)	32.150S	23	10/13	1.82 ± 0.52
		2 – Calamuchita (CaSt)	32.067S	11	8/3	2.43 ± 0.44
		3 – Setúbal Shallow (SSL)	31.567S	9	8/1	2.02 ± 0.32
		4 – Colastiné (CRi)	31.617S	28	13/15	1.36 ± 0.18
		5 – Las Pencas (PeSt)	32.283S	17	15/2	1.82 ± 0.33
		6 – Doll (DSt)	32.300S	14	9/5	1.73 ± 0.40
		7 – Paraná (PRi)	32.967S	15	3/12	1.49 ± 0.22
		8 – Areco (ASSt)	34.067S	54	5/49	2.18 ± 0.57
		9 – Martín García (IMGS)	34.200S	45	26/19	1.78 ± 0.34
		10 – Anchorena (ANSt)	34.550S	17	9/8	1.71 ± 0.39
		11 – Adrogué (ADSt)	34.650S	11	4/7	1.87 ± 0.46
		12 – Matanza (MRi)	34.733S	45	23/22	2.52 ± 0.52
Uruguay (US)		13 – Brazo Largo (BLRi)	33.850S	10	8/2	1.17 ± 0.50
		14 – De las Leches (DLSt)	32.083S	12	9/3	1.50 ± 0.35
		15 – Urquiza (UrSt)	32.047S	7	1/6	2.30 ± 0.74
		16 – Federación (FSt)	30.917S	74	36/38	1.75 ± 0.51
		17 – Ibaá (ISSt)	29.750S	15	12/3	2.13 ± 0.57
		18 – Ibirapuita (IPSt)	29.883S	17	11/6	2.10 ± 0.31
		19 – Palacio (PSt)	33.517S	22	7/15	1.84 ± 0.40
		20 – Lunarejo (LSt)	31.100S	18	8/10	2.17 ± 0.35
		21 – Candiotá (CaSt)	31.317S	7	2/5	1.83 ± 0.31
		22 – Amaral Ferrador (AFSt)	30.867S	19	9/10	1.95 ± 0.52
		23 – Seival (SSSt)	30.500S	11	7/4	1.81 ± 0.35
		Atlantic Ocean (AOB)	Maldonado (MaS) Punta del Este (PES)	24 – Maldonado (MSSt)	34.900S	9
25 – Punta del Este (PESt)	34.750S			13	12/1	1.12 ± 0.26
dos Patos (PMB)	Mirim (MiS) dos Patos (LPS)					



Landmarks and semilandmarks identification (+ semilandmarks):

- 1, Tip of the rostrum
- 2, Concavity of the orbital sine
- 3, Tip of the anterolateral spine
- 4, Union between the antero-lateral lobe and the first hepatic lobe
- 5, Spine of the the first hepatic lobe
- 6, Union between the 3rd hepatic lobe and the epibranchial tooth
- 7, Tip of the epibranchial tooth
- 8 +, Junction between the branchial line and the posterior of the linea aeglica lateralis;
- 9, Posterior extreme of the longitudinal dorsal line
- 10, Posterior and anterior extremes of the "bar line"
- 11, Union between the epibranchial tooth and the linea aeglica lateralis
- 12-14 + -15 + -16 +, Cervical groove
- 13, Centre-posterior extremes of the cephalothorax
- 17, Anterior vertices of the areola

Figure 2. The 13 landmarks (black circles) and four semi-landmarks (white circles) representing the carapace shape of *Aegla uruguayana*.

specimens, including the growth variability for each moult cycle. Each interval corresponded to the mean size of each moult period.

STATISTICAL ANALYSIS

An exploratory ordination was obtained via an analysis of the RWs with TPSRELW (Rohlf, 2007). The results of this analysis identified associations between the sub-basins. Based on these results, the populations were grouped by sub-basin: Mirim (MiS), dos Patos (LS), Paraná (PS), Rio de la Plata (RPS), Uruguay (US), Maldonado (MaS), and Punta del Este (PES) (Fig. 1).

The size variation between sampled aeglids from all populations was analyzed by analysis of variance and Tukey's post-hoc test (Q value), whereas shape variation was analyzed by multivariate analysis of variance and linear discriminant analysis (LDA). For the analysis of all specimens considered together, an LDA of the residual values of partial warps and a uniform component matrix from the regression of the shape variables on size was performed. A dissimilarity analysis (unweighted pair group method with arithmetic mean) was performed based on the Procrustes distance of the consensus configuration for each population (NTSYS (Rohlf, 2007)). Finally a two-block partial least squares method (Rohlf, 2006) with 999 permutations was performed on the shape-geographical coordinates and shape-environmental data to identify the pattern of covariation between these variables.

RESULTS

SIZE, SEXUAL DIMORPHISM, AND ALLOMETRY

The carapace length of the aeglids ranged between 1.15 and 3.96 cm. The animals from the UrSt popula-

tion were the largest size, and those from the CRi, FSt, and AFSt populations were the smallest. The mean size varied between 1.36 cm for the CRi population and 2.52 cm for the MRi population. The populations from the small streams on the Atlantic Ocean slope differed significantly in carapace size from those of the other basins (LPB-AOB Q : -0.355, P = 0.000005; PMB-AOB Q : 0.369, P = 0.000026). Moreover, the size of the aeglids from the MaS (Maldonado Sub-basin) differed from that of the other populations grouped into sub-basins (MaS-RPS Q : -0.429, P < 0.000001; MaS-PS Q : 0.328, P = 0.000032; MaS-LS Q : 0.371, P = 0.000234; MaS-US Q : 0.330, P = 0.000033). Juveniles represented 34% of all individuals, and the ratio of males to females (M/F) was 0.89.

Only three populations (DSt, ASt, and IMGS) showed significant sexual dimorphism (Hotelling's test, DS 1.83, P = 0.0360; ASt 0.44, P = 0.0027; IMGS 0.47, P = 0.0083). The shapes of males and females were similar in the remaining populations.

The analysis of size and shape in *A. uruguayana* yielded evidence for ontogenetic allometry. Based on this analysis, size ranges were defined to eliminate those effects. Within each size range, the allometric effects were not statistically significant (see Appendix, Table A2). This approach allowed us to compare the carapace shape from each size category between populations without requiring any correction for allometric effects.

SHAPE DIFFERENCES AMONG SUB-BASINS

The exploratory analysis of the RWs in each size range showed a pattern that separated the basins (i.e. LPB, AOB, and PMB) into sub-basins based on the principal rivers and lagoons within each basin (PS, US, RPS, LS, MiS, MaS, PES) (Fig. 1).

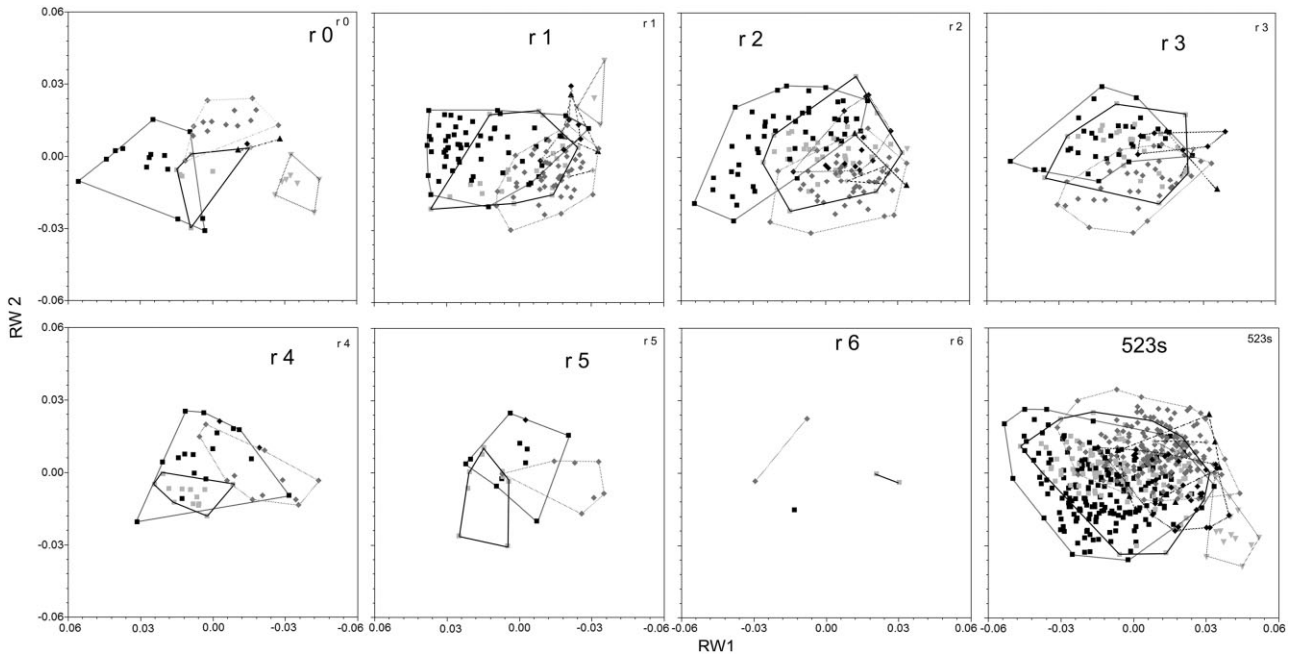


Figure 3. Relative warps of the carapace of *Aegla uruguayana* in each range grouped by basin and sub-basin. The explained percentage variation of the two first relative warps (e.g. RW1 and RW2) for each size range: r0 (45.16–15.22%); r1 (40.85–13.80%); r2 (33.91–16.72%); r3 (30.90–13.82%); r4 (31.24–15.53%); r5 (32.89–17.43%); r6 (61.41–18.99%). Sub-basins, black diamond: LPS; black up triangle: MaS; light-grey diamond: MiS; light-grey down triangle: PES, black square: PS; light-grey square: RPS; grey diamond: US.

The relationship between carapace shapes within each size category defined a pattern along a spatial gradient related to the geographical location of *A. uruguayana* populations. Similarities in shape were found among the PS, RPS, and US populations (La Plata Basin). Shape similarities were also found between the LS and MiS populations (Lagoa dos Patos Basin). The MaS populations differed in shape from the other populations (Atlantic Ocean Basin).

Sizes ranging from r0 to r3 showed a similar carapace shape pattern, in which the aeglids from the RPS fell between those from PS, and US. By contrast, sizes ranging from 4 to 6 showed a pattern in which the carapace shape of PS specimens fell between those from US, and RPS (Fig. 3). For populations from other basins, the analysis of shape showed that the LS specimens were relatively similar to the US specimens. The MiS specimens were relatively divergent from those of other populations. The aeglids from AOB had even more distinctive carapace shapes based on the RW1 information (Figs 3, 4).

Several shape differences were found among specimens from sub-basins within the size ranges considered (Fig. 4, Table 2). In the PS populations,

the front region (L1–L6), separated from the posterior region by the cervical groove (L7, L12, L14–L17), was shorter and wider than the posterior region (L8–11 and L13) of the others populations. The RPS specimens exhibited a wide anterior region (L1–L6) and a large posterior region (L8–11 and 13) relative to the corresponding region of the PS specimens. The US specimens showed a more stylized carapace shape and were narrower than the other sub-basin specimens from the LPB. The MiS specimens showed a larger rostrum (L1) than those from the other basins as well as a wider front (L3, distance between the tips of the spines of the anterolateral angles of the carapace) and posterior region (L8) referring to the distal end (or vertex) of the posterior branchial area. The LS specimens showed a shorter rostrum (L1) and a narrower carapace than did the other specimens. The MaS specimens showed a larger rostrum (L1) and a narrower carapace, with the front region (L1–L6) being longer than the posterior region (L8–11 and L13). Based on these observations, the aeglids from US most resembled the specimens from the stream locations of the MiS, LS, and MaS regions in terms of RW1 (Fig. 4). The AOB (e.g. MaS) specimens were closer to the specimens from MiS and LS on the RW1 axis in all size ranges (Fig. 4).

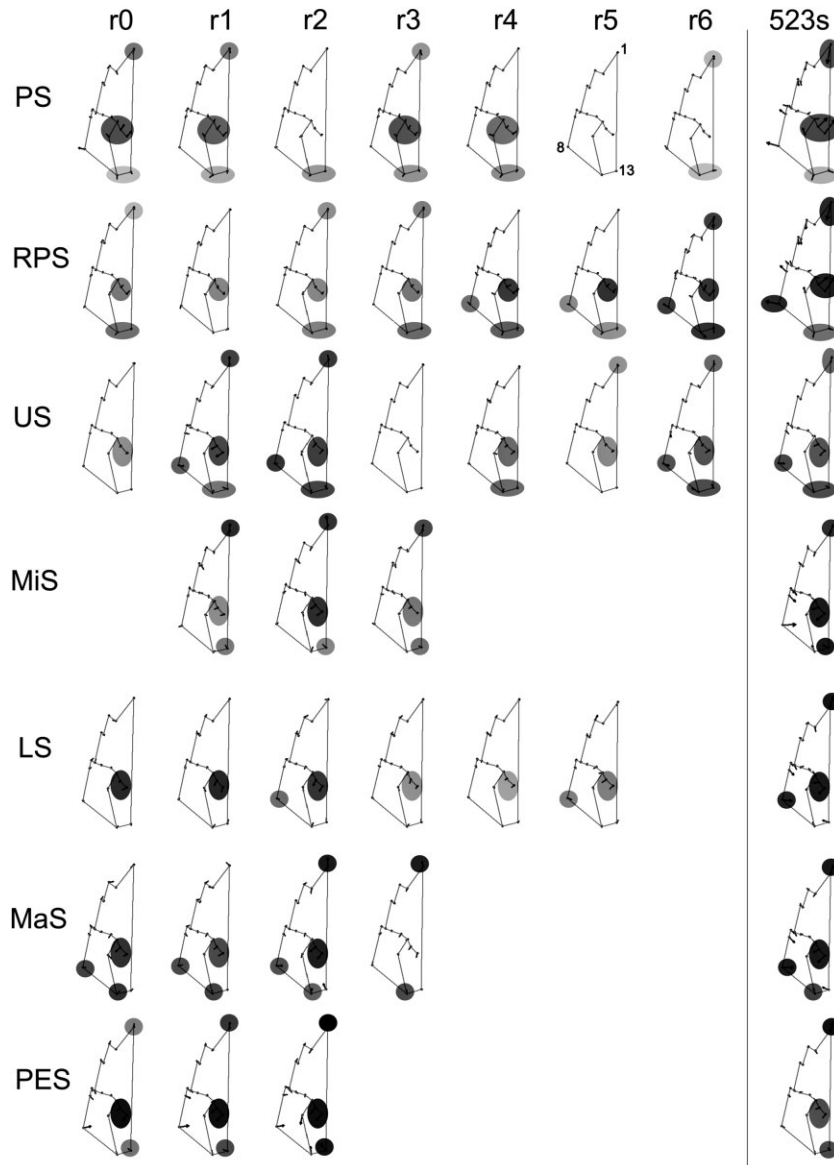


Figure 4. Deformation vectors of each size range showing the shape variation of *Aegla uruguayana* at each sub-basin. The vector direction (not magnitude) reveals a pattern and the tendency of shape variation of the individual of the different sub-basin along the seven size range (r0 to r6), and the total specimens together (523 s). Vectors are magnified ($\times 3$) to show the patterns of shape variations more clearly. An ellipse represents differences in the magnitude of shape variation. The grey tone indicates less (light grey) to more variation (dark grey). For abbreviations, see Fig. 1.

SHAPE DIFFERENCES RELATED TO GEOGRAPHICAL DISTRIBUTION

All populations from the LDA displayed a clinal pattern in each size range. The populations in the sub-basins of the La Plata Basin (e.g. PS, RPS, and US) along LD1 and LD2 had a differentiated pattern. The US populations were the link between LPB and populations from the northeastern basins (e.g. Lagoa dos Patos Basin, Atlantic Ocean Basin) (Fig. 5, Table 3).

The data from each size range were integrated and compared between all populations. This comparison showed that the pattern of variation and the total shape variation were similar within each of the size ranges. Furthermore, the consensus configurations of the crab shapes showed differences between groups. These configurations also showed a pattern in shape related to the geographical distribution of the species. This pattern was similar to that presented above (Fig. 6).

Table 2. Linear discriminant analysis (LDA) and multivariate analysis of variance (MANOVA) among sub-basins of each size range of 25 populations of *Aegla uruguayana*

LDA			MANOVA					
Range	LD1	LD2	Wilk's λ	d.f.	Ap F	Numerator d.f.	Denominator d.f.	$P_r(>F)$
0	0.751	0.177	0.0001	4	3.811	120	50.32	$= 2.892 \times 10^{-07}$
1	0.524	0.229	0.0140	5	4.880	150	534.22	$< 2.2 \times 10^{-16}$
2	0.667	0.138	0.0232	5	4.576	150	598.51	$< 2.2 \times 10^{-16}$
3	0.555	0.220	0.0118	5	3.455	150	356.21	$< 2.2 \times 10^{-16}$
4	0.5061	0.3907	0.0001	3	5.603	90	27.82	$= 1.491 \times 10^{-06}$
5*	0.6311	0.2767	–	–	–	–	–	–
6*	–	–	–	–	–	–	–	–

Ap F = approximated F ; $P_r(>F)$ = the probability of a greater F value.

*Variables are collinear. Residuals have range 23 < 30.

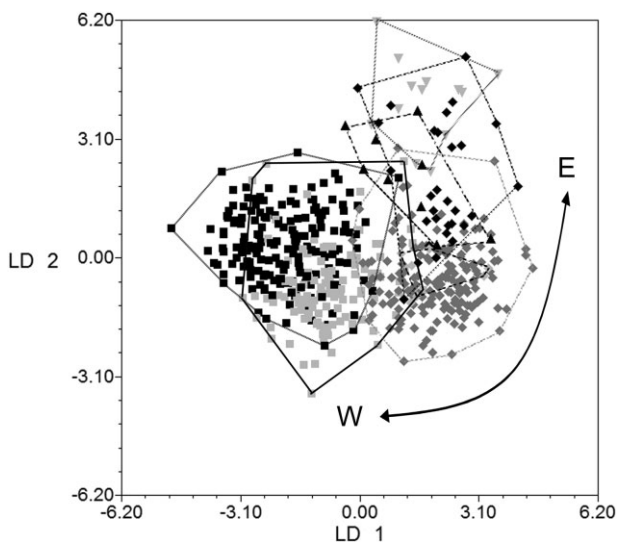


Figure 5. Linear discriminant analysis showing the pattern of the carapace shape variation of *Aegla uruguayana* ($N = 523$) in a gradient from western (W) to eastern (E), according to the sub-basin locations. Sub-basins, black diamond: LPS; black up triangle: MaS; light-grey diamond: MiS; light-grey down triangle: PES; black square: PS; light-grey square: RPS; grey diamond: US. For abbreviations, see Fig. 1.

The distance coefficients between the study populations showed a relationship to the geographical distance between the populations, grouping the populations according to their degree of proximity and/or connectivity to the river system. For example, the populations in the Paraná Sub-basin (i.e. SSL, CRi, PeSt, DSt, and PRi) formed a hierarchical group, the populations near the Rio de la Plata Sub-basin (i.e. ASt, ANSt, ADSt, and MRi) were similar, and those populations that showed a relationship to the

Uruguay Sub-basin (i.e. DLSt, UrSt, FSt, ISt, IPSt, CaSt, PSt, and LSt) were associated with IMGS. Of all the populations studied, the populations located in the extreme eastern area of the distribution of the species (i.e. PES) showed the greatest difference in their relationship to the distance coefficient.

The principal cluster was characterized by two groups: the PS–RPS sub-cluster and the LPS–MaS–MiS–other populations of RPS–US sub-cluster, leaving PES as an outgroup (Fig. 6). In the first sub-cluster, SUSt was the most peripheral (isolated) population, occurring in a distinctive habitat (i.e. a mountain stream with high dissolved oxygen, extreme temperatures, abundant areas for refuge under rocks, low diversity, and scarcity of predators). Additionally, it showed certain similarities with LSt. The CalSt and MRi specimens were relatively close, although the habitats of these populations differed in water velocity and substrate type (i.e. mountain streams versus lowland streams).

The Atlantic Ocean Basin and Lagoa dos Patos Basin specimens were similar in shape. The PES population was the most isolated of all *A. uruguayana* populations considered, located at the eastern extreme of the geographical range of the species.

The carapace shapes of *A. uruguayana* specimens studied and the geographical location of study sites showed a statistically significant covariation (Fig. 7, Table 4). The first dimension reveals a longitudinal pattern (East–West) of shape variation between populations that follows the basin–sub-basin distribution. The second dimension shows a variable combination in which a latitudinal pattern (South–North) of shape was observed. The correlations between the first and second dimensions of combination between shape and variables were statistically significant as well. Additionally, the carapace shape differences were observed among sub-basin populations agree with

Table 3. Linear discriminant analysis (LDA) and multivariate analysis of variance (MANOVA) among sub-basins and basins without separating the size of *Aegla uruguayana* from 25 populations in the distribution area

	LDA		MANOVA				
	LD1	LD2	Wilk's λ	Ap F	Numerator d.f.	Denominator d.f.	$P_r(> F)$
Basins	0.7517	0.2483	0.4096	9.21	60	982	$< 2.2 \times 10^{-16}$
Sub-basins	0.5972	0.1899	0.0477	10.80	180	2881.1	$< 2.2 \times 10^{-16}$

Ap F = approximated F ; $P_r(> F)$ = the probability of a greater F value.

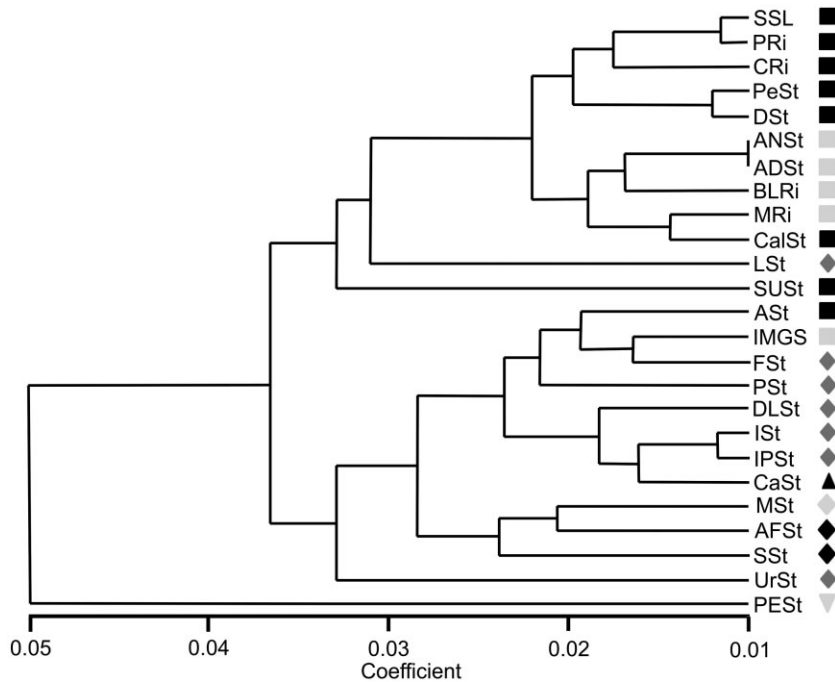


Figure 6. Unweighted pair group method with arithmetic mean analysis from Procrustes distance of consensus configurations of 25 populations showing the similarity–dissimilarity of specimen carapace shapes of *Aegla uruguayana*. Sub-basins, black diamond: LPS; black up triangle: Mas; light-grey diamond: MiS; light-grey down triangle: PES; black square: PS; light-grey square: RPS; grey diamond: US. For location abbreviations, see Fig. 1.

those observed in the range analysis (Fig. 3). However, the relationship between shape and environmental variables was not statistically significant and showed no covariation. By contrast, the correlation calculated between shape and environmental variables was statistically significant according to the permutation test (Table 5). Nevertheless, it does not appear that variations in shape were associated with environmental variables but, rather, the populations were associated more strongly with their geographical distribution (clinal variation) than with the characteristics of the studied environments (Fig. 7).

The shapes of the aeglids in the La Plata Basin showed a relationship to the proximity of the population sites and sub-basins. Within the Paraná Sub-basin, the populations were relatively similar (e.g.

SSL, DSt, PeSt, CRi, PRi, AS). The populations near the Río de la Plata Sub-basin were also relatively similar to each other (e.g. IMGS, ANSt, MRi, ADSt) and showed a pattern of gradual shape change. Furthermore, populations with a relationship to the Uruguay Sub-basin formed a group (e.g. UrSt, ISt, FSt, IPSt, CaSt, DLSt, PSt, LSt). The animals that showed a more divergent shape pattern were those that inhabited mountain areas and those that were separated from the remaining populations but maintained a connection to the Paraná Sub-basin (e.g. SUSSt, CalSt).

DISCUSSION

A clinal pattern was detected for shape variation but not for size variation in *A. uruguayana*. The pattern

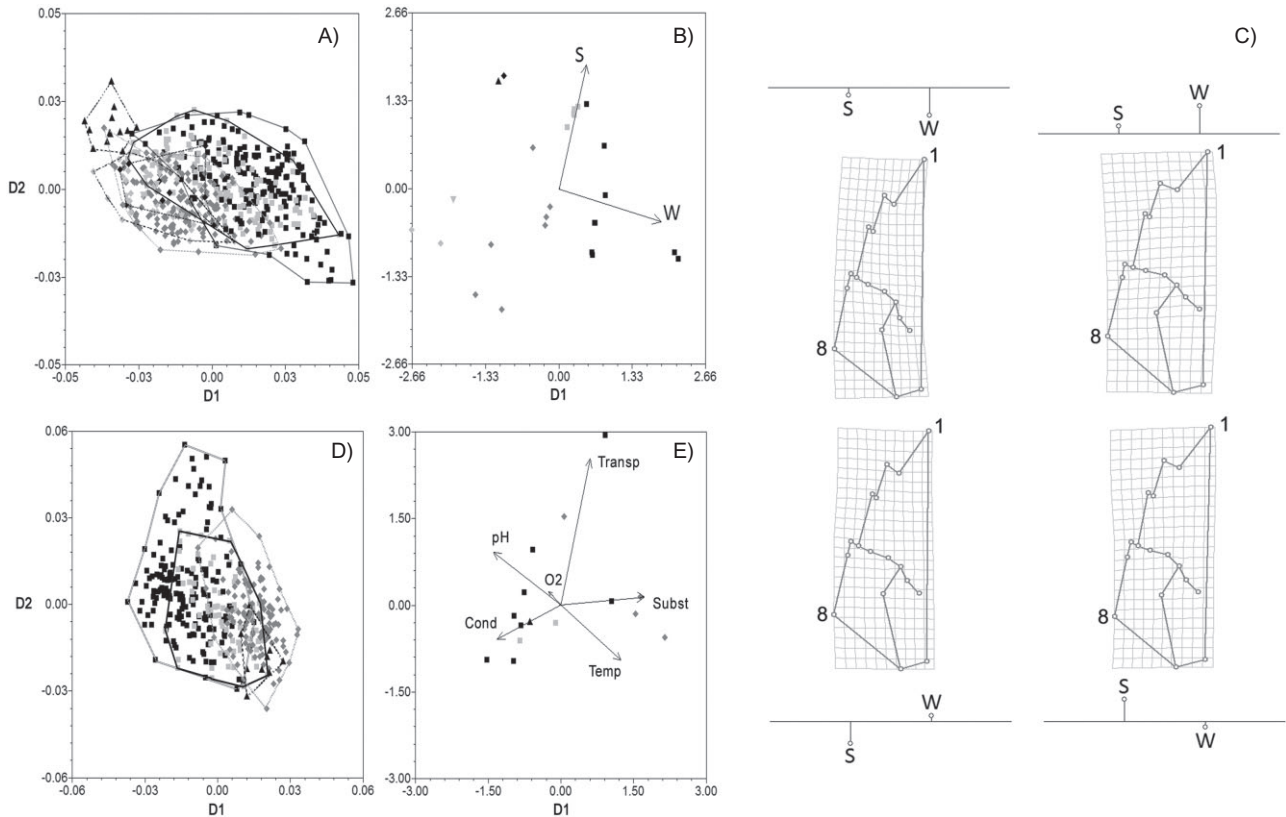


Figure 7. Shape (A) and geographical coordinate (B) projections of covariation (S, South; W, West). Deformations grid of carapace shape according to the population distribution (C), shape (D) and environmental variable (E) projections of covariation in *Aegla uruguayana* (Transp = Transparency, Subst = Substrate type, Cond = Conductivity, Temp = Temperature, O₂ = dissolved oxygen, and pH). Sub-basins, black diamond: LPS; black up triangle: MaS; light-grey diamond: MiS; light-grey down triangle: PES; black square: PS; light-grey square: RPS; grey diamond: US.

Table 4. Partial least squares analysis among shapes of 25 populations of *Aegla uruguayana* and their distribution position

Var – cov							Correlations			
L var	Cov	Cov ²	% Exp total cov ²	SV obs	Count	%	L var	robs	Count	%
D1	0.0128	0.0002	94.29	0.943	1	0.10	D1	0.654	1	0.10
D2	0.0031	0.0001	5.71	0.057	1000	100	D2	0.346	1	0.10

Permutation tests with 999 random permutations ($N = 523$). L var, linear variation dimensions; Cov, covariation value observed; Cov², squared covariation; % Exp total cov², percentage of the total covariance explained; SV obs, singular values observed; robs, r value observed; Count, count of the number of random permutations; %, percentage of the number of random permutations.

of phenotypic variation identified in the present study was related to the geographical distribution of the species and the history of geographical connections in a geoclimatic framework. Differences in shape could be related to drift, more so than selection, when a population is divided sufficiently (Irwin, 2012), as in this case, in which geographical distribution was

more recently developed. The analysis of shape variation showed that the LPB specimens were similar to each other and differed from the aeglids inhabiting the other basins. Generally, the analyses of the models of carapace shape identified two principal patterns, related to populations connectivity and the history of each population in recent times. The US

Table 5. Partial least squares analysis among shapes of 17 populations of *Aegla uruguayana* and the environmental variables

Var – cov							Correlations			
L var	Cov	Cov ²	% Exp total cov ²	SV obs	Count	%	L var	Robs	Count	%
D1	0.0110	0.00012	55.21	0.552	343	34.30	1	0.623	1	0.10%
D2	0.0080	0.00007	30.07	0.301	121	12.10	2	0.553	1	0.10%

Permutation tests with 999 random permutations ($N = 367$). L var, linear variation dimensions; Cov, covariation value observed; Cov², squared covariation; % Exp total cov², percentage of the total covariance explained; SV obs, singular values observed; robs, r value observed; Count, count of the number of random permutations; %, percentage of the number of random permutations percentage.

specimens from the LPB were linked to the PMB and AOB crab populations. Additionally, the differences in carapace shape between populations from more widely separated basins or sub-basins were greater than those for the specimens whose populations served to integrate a meta-population, or were located in the same basin, or in nearby habitats. Nevertheless, the US specimens showed a shape similar to that of the specimens from the other sub-basins.

According to recent studies, Giri *et al.* (2014) found an inverse correlation between hydrographic distance and gene flow among *A. uruguayana* populations. This evidence suggests isolation by distance among the populations with higher genetic differentiations (highest H_E and P values) and in populations from different sub-basins than in populations from same sub-basin. In another study, Gotelli *et al.* (1985) found a clinal pattern in the carapace morphology of the crab *Trapezia cristulipes* (Stimpson, 1860) similar to that observed for *A. uruguayana*, including variations in the branchial region, rostrum, and propodus. Moreover, Rufino *et al.* (2004) found differences in portunoid crabs between Atlantic and Mediterranean populations that displayed restricted genetic interchange. Such factors could explain the results found for the specimens from the AOB and PMB. Furthermore, intraspecific differences have been found among populations of freshwater crabs *Potamonautes perlatus* (Milne-Edwards, 1837). In southern Africa, freshwater crabs of the genus *Potamonautes* show a biogeographical size gradient (Daniels *et al.*, 2002). In the present study, however, size differences in the aeglid populations were not clinally ordered, and the small variations observed lacked any biogeographical pattern.

Additionally, isolation events resulting from marine transgressions have been reported in the fauna. These events have disrupted and interrupted the process of gene flow between populations that had previously been connected, as in the case of *Callinectes bellicosus* (Stimpson, 1859) (Pfeiler *et al.*,

2005). Furthermore, several studies present evidence of rapid re-colonization by decapods after periods of intense glaciation (i.e. European freshwater crayfish populations that inhabited micro-refuges in the Alps and thus survived glaciations) (Hewitt, 1999; Trontelj *et al.*, 2005; Schubart & Huber, 2006; Chiesa *et al.*, 2011). From this perspective, studies of the freshwater crabs *Potamonautes parvispina* (Stewart, 1997) and *Potamonautes sidneyi* (Rathbun, 1904) have demonstrated that the similar genetic structure of populations from different basins or sub-basins was associated with the rapid dispersal of these crabs after geoclimatic events (Stewart, 1997; Daniels *et al.*, 1998). Considering studies of *A. uruguayana* populations across the La Plata system (Uruguay and Parana Sub-basins), gene flow among populations is low ($F_{ST} = 0.3583$) and similar to the total migration rate ($N_M = 0.4477$), being genetically similar to the closest populations according to hydrographic distances (Giri *et al.*, 2014). Studies of *P. perlatus* (Daniels *et al.*, 2006) have reported regional phylopatric patterns among individuals from different populations. Molecular differences occurring between populations separated by a mountain range were estimated to have originated in the Miocene (6.3–24 Mya). This period was characterized by substantial climate changes in Africa and by several marine transgressions (Daniels *et al.*, 1998), which also affected South America (Lundberg *et al.*, 1998). Dos Reis *et al.* (2002) concur by highlighting that the shape of rodent cranial morphology is associated with geological and climatic events occurring in the Miocene/Pliocene/Pleistocene (1.8–23 Mya), which caused periods of population separation.

The shape pattern observed in *A. uruguayana* could indicate that the interruption of connections between basin populations (or meta-populations) occurred recently. As noted, the history of freshwater basins in South America is marked by substantial geomorphological events. For this reason, these systems are highly dynamic. The most important

geomorphological event that could have directly affected the distribution of *A. uruguayana* was the great marine transgression of the late Miocene (10–11.8 Mya). This transgression formed the Paranaense Sea, which extended to the region bordering Argentina, Paraguay, and Bolivia (north and north-west of 17° S) and was bounded by the massif of the Sierras Pampeanas and the Brazilian Shield (Lundberg *et al.*, 1998). During this period, the principal rivers of the La Plata Basin did not flow along their current waterways, and the region was very different from its present arrangement. Subsequently, the region of the Atlantic Ocean Basin was also victim to several marine transgressions (Martínez & Del Río, 2002), alternating between glaciations that affected northern Patagonia. Correlations between these events and the Pampean loess are plausible, although additional studies are necessary (Rutter *et al.*, 2012). Pérez-Losada *et al.* (2004) noted that the clade to which *A. uruguayana* belongs was formed between 10.7 ± 1.1 Myr, after the retreat of the Paranaense Sea. Although our hypothesis is that populations of *A. uruguayana*, or of its ancestral species, were present prior to the marine transgressions, individuals belonging to this species could invade an extensive area similar to that of the present distribution. Certain populations were subsequently isolated in sub-basins, producing the features of the present clinal pattern.

Of the previous geomorphological events discussed above, the event considered most relevant to the current distribution of *A. uruguayana* is the marine transgression of the Late Miocene. If *A. uruguayana* or its ancestral species was present, we hypothesize that these populations were forced to retreat to the edge of their distribution (e.g. to the aquatic environments of the eastern Sierras Pampeanas massif; namely the SUS and CaSt populations) and those of the western Brazilian Shield (i.e. the Negro River and Ibicui River). In these environments, the phylogenetic relationships presented by Pérez-Losada *et al.* (2004), in which the clade *A. uruguayana* is included, would include species found at the edge of the transgressions. In view of this and according to the species concept, we could view certain *A. uruguayana* populations in terms of a clinal model (Coyne & Orr, 2004; Wiens, 2004; Irwin, 2012), with interruptions of gene flow and the isolation of populations.

In particular, the analyses of the LPB population showed that the carapace shape of the RPS specimens was close to the consensus configuration. This finding is consistent with the identification of the geographical centre of the distribution of the species. In view of this information, the shape of the aeglids from the La Plata River might have resulted from the convergence of animals originating in the Paraná and Uruguay Rivers (e.g. PS and US), as well as from the differ-

ences between the habitats. The PS includes the Paraná River, which represents 75% of the discharge of the entire system. A comparison of the PS and US specimens shows that the PS specimens were closer in shape to those of the RPS. Most likely, the influence of the PS was greater than that of the US. These observations serve to define two principal shape patterns: those of PS–RPS and those of the other populations. A reconstruction of geomorphological events from the present to the past marine transgressions serves to define a hypothesis about the origin of *A. uruguayana* in the basins considered in the present study. During the most recent transgression (the Querandinense transgression, 4000 to 6000 years BP), the sea invaded the La Plata Basin as much as 300 km upstream, through the channel of the Paraná River and up to Guauguaychú through the Uruguay River, including the link between the Negro River and Uruguay River (Aceñolaza, 2004). The intermediate shape characteristics of the La Plata River specimens could be a result of the effects of the recent transgression (Ringuelet, 1956).

According to the same argument outlined by Daniels *et al.* (2006) regarding the importance of geomorphological history, the river basins of southern South America could yield information that helped to shed light on the evolutionary history of the aeglids. The history of these rivers was shaped by various events occurring subsequent to the separation of Africa and America. If we place the origin of the aeglids at approximately 74 Mya (upper Cretaceous) (Feldmann, 1984, 1986), the principal South American geomorphological and climatic events to consider in this context are the uplift of the Andes chain, the megadome formation in eastern South America and at least six marine transgressions (Potter, 1997; Lundberg *et al.*, 1998; Ribeiro, 2006).

The environmental characteristics of certain study sites differed and the body shape similarities shown were best explained by proximity of basins, rivers or particular sampling sites. This evidence supports the hypothesis that the observed shape features, which show a clinal pattern, are a product of the recent distribution of the studied meta-populations of *A. uruguayana*. The analysis of relationships involving shape indicated that the principal differences in shape were related to sub-basin origin and that the effects of the environmental conditions within each sub-basin were relatively insignificant. Similarly, other species of Aeglididae with wide distributions, such as *Aegla platensis* or *Aegla neuquensis*, could show the same relationship between shape, origin and environment. A pattern of shape variation according to water velocity was reported by Giri & Loy (2008) for *A. neuquensis* and Zimmermann *et al.* (2012) for *Macrobrachium australe*. In the present study,

considering the whole population, we did not find any environmental pattern in *A. uruguayana*. Thus, in agreement with the findings of Zimmermann *et al.* (2012), *A. uruguayana* could be plastic considering the wide distribution and different environment for this species. The distribution of the population and the environmental conditions of each site (i.e. the local physical-chemical variables) also influenced the shape patterns among populations observed in different zoological groups, such as vervet monkeys in Africa (Cardini *et al.*, 2007) and whitefish *Coregonus* sp. (Vonlanthen *et al.*, 2009). However, in the present study, the environmental condition was not as strong an influence as the geographical position of the site. In this context, the migrations among populations or the demes of the meta-population (Sultan & Spencer, 2002) are reflected by the similarity of shape of individuals of nearby populations, and this pattern was registered by Giri *et al.* (2014) in molecular studies (see above). The combined effect of the distribution (clinal) and environment characteristics would present, in a subsequent stage, a slight degree of phenotypic variation correlated with ecogeographical variation. Hence, gradual phenotypic variation would rise along a given ecogeographical gradient. This situation could correspond to the concept of a 'smooth cline', as exemplified by a species without marked subspecies (Salmon, 2002).

The current scenario involving the distribution of *A. uruguayana* populations may indicate that shape differences and phenotypic variations are a result of recent meta-population distribution. This process may be magnified by the geographical isolation of the basins, differences in environmental characteristics, and the low dispersal ability of the species.

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APPENDIX

Table A1. Environmental variables that characterize the sampled sites *Aegla uruguayana* (substrate type: 1, silt and sand; 2, gravel and coarse gravel; 3, cobble and boulder)

Population	pH	Temperature (°C)	O ₂ (mg L ⁻¹)	Transparency (cm)	Conductivity (µS cm ⁻¹)	Substrate
1 – Segunda Usina Stream	7.25	19.00	6.80	80.00	172.00	3
3 – Setúbal Shallow lake	7.50	21.90	7.30	20.00	100.00	1
4 – Colastiné River	7.50	21.90	7.30	30.00	100.00	1
5 – Las Pencas Stream	7.25	19.00	9.46	50.00	600.00	3
6 – Doll Stream	7.25	19.00	9.46	38.00	600.00	3
7 – Paraná River	7.25	19.00	6.80	30.00	107.00	1
8 – Areco Stream	7.25	19.00	6.80	25.00	700.00	2
9 – Streams in Martin Garcia Island	7.18	21.20	6.60	39.00	163.00	1
10 – Anchorena Site	7.00	18.00	8.10	25.00	136.00	1
11 – Adrogué Site	7.00	18.00	8.10	25.00	136.00	1
13 – Brazo Largo River	7.06	23.30	5.73	58.00	223.00	1
14 – De las Leches Stream	6.90	21.50	7.10	40.00	65.00	3
15 – Urquiza Stream	7.10	21.50	7.70	45.00	65.00	3
16 – Federación Stream	7.10	21.50	7.90	40.00	69.00	3
19 – Palacio Site	7.70	21.50	8.40	50.00	68.00	3
24 – Maldonado Stream	7.30	18.00	5.00	25.00	97.80	2

Table A2. Size ranges (*r*) indicating the inferior and superior limit of each range (Lim Inf, Lim Sup), total number (*N*), number of males and females of *Aegla uruguayana*, and allometry tests of each size range to evaluate the relationship between shape and size

Range (<i>r</i>)	Lim Inf (cm)	Lim Sup (cm)	<i>N</i>	Males	Females	Generalized Goodall's <i>F</i> -test (<i>F</i>)	d.f.	Permutation tests (<i>P</i>)
0	0.71	1.14	46	29	17	0.8497	30–1320	0.491
1	1.15	1.58	142	62	80	1.6768	30–4200	0.123
2	1.59	2.02	155	70	85	1.9930	30–4590	0.066
3	2.03	2.46	106	51	55	1.0066	30–3120	0.421
4	2.47	2.90	42	25	17	1.1657	30–1200	0.271
5	2.91	3.34	27	18	9	1.1869	30–750	0.304
6	3.35	3.96	5	4	1	–	–	–