

# Revision of Manuscript bg-2017-390

## ‘Impact of climate and hydrochemistry on shape variation – a case study on Neotropical cytheroidean Ostracoda’

5 As indicated in our reply to the reviewer comments we have revised our manuscript carefully.

### Revisions according to Reviewer #1

In particular, reviewer’s major concern is that our analyses focused on PLS analyses and not (as usual) MANOVA/MANCOVA or Procrustes ANOVA.

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- We have carefully checked our introduction. It is indicated in p2 lines 4-10 that the biogeographical variation is known, but the present study focus on regional differences that have not been investigated, yet. Therefore, we have applied two-block partial least squares analyses that enable to study identify the morphological characteristics and environmental variables that contribute most to the relationship (see p2 line 9-10).

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Referee 1 also criticizes that we performed regression analyses on each relative warp.

- The respective section was rephrased in order to highlight that this approach enables direct evaluation which environmental factors are associated with which kind of shape variation. See p4 line 29 -p5 line 3.

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“In addition, further inspection on the result of PLS analysis is recommended. The authors emphasize the similarity between Florida and Brazil based on the plot of RW1 vs RW2, but Fig. 5 indicated the similarity between Florida and Mexico. Graphical presentation of shape deformation indicated by PLS singular axis of shape variables should be added for further discussion. Loadings of environmental variables in PLS singular axis will be helpful for understanding the effects of climatic and hydrochemical factors. The authors reported first PLS singular axes, but second PLS singular axes might also indicate relationship between shape and environmental factors.”

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- We have included a sentence about the results of the second PLS axis in the manuscript (p 6 line 26-27).
- As suggested we providing the detailed results in the supplements because of the minor relationship between morphology and environment.
- Also, we added the loading values into the supplementary material. We formulated the selection criteria for the warps more clearly in the manuscript. Accordingly, we added the regression analysis for RW3 of females LV. The values will be changed in the table.

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“Discussion in the paper descriptively indicates that there are morphological differences that possibly relates to environmental variables, but there are little discussions on why shape of ostracod valves differ depending on environments (e.g. are there any

functional meanings? or merely due to physicochemical consequences?) and how ostracods respond to environmental changes. It is impossible to achieve definitive conclusions in the paper, but at least proposing some hypotheses is required.”

- 5
- We will extend our discussion according to the referee’s comments, including the consideration if the shape changes might be the result of functional advantages or if they are the result of physiological processes. See p 7 line 28 – p 8 line 5 and page 10 line 4 -14.

Page 3 Line 1: The authors focus on “the causes for the regional differences”, so how the factors cause the morphological differences should be more discussed in Discussion.

- 10
- See above. We extended the discussion with respect to explain morphological differences.

Line 20: Add explanation more about “water sampling”.

- The information were added.

Page 6 Line 18: Show the result of statistical testing of the “clear relationships” in the PLS analyses. Statistical testing is possible by using resampling technique, such as permutation test.

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- We have applied permutation tests on the PLS results. Results are given at p6 line 20-25.

Lines 27–28: Table 1 does not show that higher warps (the authors mean RW4, RW5, etc.?) have minor influence on shape variation. In this paragraph, do the authors show the results of relative warp analysis?

- 20
- This paragraph is about the PLS analysis. We have reformulated it to avoid further confusion.

Page 8 Lines 5 & 23: The authors only noted that the reasons why shape differences relate to environmental factors are not or poorly understood. However, at least proposing some hypotheses is required.

- 25
- See above. As indicated we have inserted some hypotheses.

Technical corrections were done according to the referee’s comments.

### **Revisions according to Reviewer #2**

30 His concerns followed referee #1 demanding some hypothetical interpretations about the mechanisms behind the relationship between shape of ostracod valves and environmental parameters.

- See above. The discussion was extended and includes some hypothetical interpretations about the mechanisms behind the relationship between shape of ostracod valves and environmental parameters.

### Revisions according to Reviewer #3

- 5 His concerns are similar to the both other referees and refer to missing explanation for the mechanisms that drive shape variation. He/she suggests adding complementary studies that show relationship between environmental variables and valve shape. It is also noted that we should describe the effects of change in morphological characteristics on ecological significance such as effects on production and behavior.
- 10
- We have checked our discussion carefully and hypothesized possible mechanisms (see reply to referee #1, functional vs. physiological meaning).
  - Since information on ostracod biology is quite fragmentary we have not found for appropriate studies that could have contribute to the present study.
- 15 L22-23: Please write about storing condition. In a freezer?
- Information were added.
- [P9] L22-L2(P10): Authors guess the relationships between genetic differences and carapace shape at the discussion part,
- 20 lengthy. It is not good to describe lengthy about what is not contained your data. Please delete this speculative sentences.
- Unnecessary information was deleted to shorten the discussion.
- Fig. 3-5 The symbol of data from “Punta Laguna” should be changed.
- 25
- We reconsidered the request of the reviewer and decided for reasons of consistency not to follow him/her. The manuscript focuses on regions not on populations. The population “Punta Laguna” is mentioned since there are two morphotypes which causes an overlap of the Floridian and Mexican morphospace. However, this is sufficiently discussed in our other studies which are cited.



# Impact of climate and hydrochemistry on shape variation – a case study on Neotropical cytheroidean Ostracoda

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**Abstract.** How environmental change affects a species' phenotype is crucial not only for taxonomy and biodiversity assessments but also for ~~their~~its application as (paleo-)ecological indicators. Previous investigations addressing the impact of climate and hydrochemical regime on ostracod valve morphology have yielded ~~quite~~ contrasting results. Frequently identified ecological factors influencing carapace shape are salinity, cation and sulphate concentrations and alkalinity. Here, we present a thorough approach integrating data from carapace outline and surface details of the ubiquitous Neotropical cytheroidean ostracod species *Cytheridella ilosvayi*, as well as several climatic and hydrochemical variables, in order to investigate a potential link between morphology and environmental conditions. A previous study lately demonstrated considerable biogeographical variation in valve morphology among Floridian, Mexican and Brazilian populations of this species. We hypothesize that the climatic differences between the regions it inhabits and associated differences in hydrochemical regimes have influenced valve morphology and eventually led to biogeographically distinctive groups. Generalized least-squares Procrustes Analyses based on outline and structural features were applied to left and right valves of adult females and males. The analyses identified relative carapace length and shape symmetry as most important morphological characteristics representing shape ~~change~~differences across all datasets. Two-block partial least-squares analyses and multiple regressions indicate strong relationships between morphological and environmental variables, specifically with temperature seasonality, annual precipitation and chloride and sulphate concentrations. We hypothesize that increased temperature seasonality slowed down growth rates during colder months, potentially triggering the development of shortened valves with well-developed brood pouches. Differences in chloride and sulphate concentrations, related to fluctuations in precipitation, are considered to affect valve development via controlling osmoregulation and carapace calcification. These factors represent hitherto unknown drivers for ostracod ecophenotypy and ~~emphasize~~ that environmental predictors for morphological variability are not consistent across non-marine ostracods.

## 1 Introduction

Understanding how species respond to environmental change is crucial for their application as proxies for past climate fluctuations as well as forecasting future dynamics and distribution of species. Morphological diversity represents a key character for the interpretation of faunal changes (Wagner and Erwin, 2006) and ecological shifts (Mahler et al., 2010) and urges discussions about speciation and extinction processes through time (e.g., Ciampaglio, 2004). Differences in shape and size among species have been shown to relate with changes of environmental parameter, in particular, differences in temperature across various clades (e.g., Loehr et al., 2010; Maan and Seehausen, 2011; Danner and Greenberg, 2015). Within freshwater invertebrates, ecophenotypic response has been documented for a variety of species, both recent and fossil (e.g., Hellberg et al., 2001; Zieritz and Aldridge, 2009; Inoue et al., 2013; Neubauer et al., 2013; Clewing et al., 2015).

Ostracods represent a ~~model~~ popular proxy group for climate and ecosystem changes due to their occurrence in various habitats, ranging from most inland waters, to marine and interstitial, and even (semi-)terrestrial environments (e.g., Horne, 2004). Their distribution is controlled by ecological factors such as salinity, temperature, and ion composition of the ambient water, etc. (e.g., Ruiz et al., 2013). The study of ecophenotypical variation in response to environmental change (Anadón et al., 2002; Frenzel et al., 2012; Fürstenberg et al., 2015; van der Meeren et al., 2010) demonstrates a further approach using them for palaeoenvironmental studies. Due to their calcitic valves, they have an excellent fossil record and are utilized as palaeoenvironmental and biostratigraphic indicators (Anadón et al., 2002). A number of studies has shown that ornamentation, nodding, sieve pore shape, and carapace size are linked to environmental factors, e.g., salinity, temperature, water depth and nutrient availability (van Harten, 1975; Yin et al., 1999; Majoran et al., 2000; van Harten, 2000; Anadón et al., 2002; Frenzel and Boomer, 2005; Medley et al., 2007; Marco-Barba et al., 2013; Meyer et al., 2016; Boomer et al., 2017). Especially with the rise of morphometric techniques, investigations also dealing with carapace shape variation in relation to environmental variables have increased (Yin et al., 1999; Baltanas et al., 2002; Baltanas et al., 2003; van der Meeren et al., 2010; Ramos et al., 2017; Grossi et al., 2017). Yet, the use of morphological data, even those based on morphometric analyses (Baltanas et al., 2002; Baltanas et al., 2003; van der Meeren et al., 2010; Grossi et al., 2017), has been restricted to either landmark-based or outline-based studies but have rarely used a combination of both (e.g., Ramos et al., 2017). Few studies integrate geographic gradients into their statistical analyses and corresponding climate variables or a reduced number of predictor variables. Moreover, shape-environment relationships are commonly identified based on simple linear regressions or qualitative observations on multivariate ordination methods.

Here, we apply a thorough approach integrating data from carapace outline and surface details, as well as several climatic and hydrochemical variables, in order to investigate a potential link between morphology and environmental conditions. Subject of study are valves of the Neotropical cytheroidean ostracod species *Cytheridella ilosvayi* Daday, 1905. Wrozyna et al. (2016)

and Wrozyzna et al. (under review) lately demonstrated considerable biogeographical variation in valve morphology among Floridian, Mexican and Brazilian populations of that species. Morphological differences in populations of *C. ilosvayi* are discernible for both valves and appendages, for adult and juvenile (A-1 to A-3) stages and across sexes, suggesting that morphological divergence is a result of long-term biogeographic isolation (Wrozyzna et al., submitted). While the morphological aspects of the biogeographic variability in *C. ilosvayi* are well understood, the causes for the regional differences have not been investigated. We hypothesize that the climatic differences between the regions inhabited by *Cytheridella ilosvayi* and associated differences in hydrochemical regimes have influenced valve morphology and finally led to biogeographically distinctive groups. We apply two-block partial least squares analyses and multiple regression analyses, in order to test for covariation between the two sets of parameters (morphology, environment) and to identify the morphological characteristics and environmental variables that contribute most to the relationship. ~~This approach enables to directly evaluate the shape variations and associated environmental factors.~~

## 2 Material and Methods

### 2.1 Material

Specimens of *C. ilosvayi* derive from several sampling campaigns in Florida, Mexico and Brazil during 2009–2015 (Fig. 1). A detailed list of the sampled localities is available in [Appendix-Supplementary Table S1](#). Only adult valves were utilized in this study, providing a sufficient number of left and right valves ~~across-for~~ both sexes. Right and left valves were investigated separately due to dimorphism in size and shape (Wrozyzna et al., 2014). Beyond that, females and males were analyzed separately because a large part of within-valve variation has been shown to depend on sexual differences (such as the presence of brood pouches in females; Wrozyzna et al., 2016).

### 2.2 Predictor variables

Altogether, 15 variables were included in the analyses. Simultaneously to water sampling, field variables (electrical conductivity, water temperature and pH) were measured *in situ* at all sample sites using a WTW multi-sensor probe (Multi 3420 Set C). Water samples were ~~taken with plastic bottles and~~ promptly filtrated using a syringe filter with a filter pore size of 0.45 µm and stored ~~in a freezer~~ until analysis. Major ions were measured at the laboratory center of Joanneum Research in Graz by ion chromatography (Dionex ICS-3000). As the variables measured per sampling station only provide a snapshot of the local ecological conditions, the set of variables was supplemented with bioclimatic data from the WorldCLIM database (WorldClim, 2017), providing data on monthly to yearly scales. From the many variables available we included annual mean temperature [°C] (BIO1), mean diurnal range [°C] (BIO2; mean of monthly maximum-minimum temperature), temperature seasonality [°C] (BIO4; standard deviation \*100), annual precipitation [mm] (BIO12) and precipitation seasonality (BIO15;

coefficient of variation), each with a spatial resolution of 30". We chose not to include all bioclimatic variables because many of them are highly correlated, causing issues for the regressions. Bioclimatic variables and occurrence data were linked in ESRI ArcGIS v. 10.4 with the tool "Extract Multi Values to Points". Environmental variables are provided in Appendix S2.

## 5 2.3 Methods

### 2.3.1 Generalized Procrustes Analysis

Valve morphology was captured using a combination of landmarks and semilandmarks. Eight landmarks (LM) were chosen to characterize anterior pore tubuli (LM 1–5, type-I) and the dorsal dip point of the posterior curvature (LM 6, type-II), as well as to delimitate maximum anterior and posterior curvatures (LM 7–8, type-III). Carapace outline was defined by two curves between LM 7 and 8, each comprising 30 equidistantly spaced semilandmarks (see also Wroczynna et al., 2016). All points were set on digitized SEM images using the program TpsDig v. 2.17 (Rohlf, 2013). The sliders file determining sliding direction of the semilandmarks during the Procrustes alignment was created in TpsUtil v. 1.58 (Rohlf, 2015). A Generalized least-squares Procrustes Analysis, computing consensus configuration, partial warps and relative warps (RW), was performed in TpsRelw v. 1.65 (Rohlf, 2016). Thin-plate spline deformation grids were used to visualize deviations of selected configurations from the mean and to identify morphological characteristics that account for differences among geographic regions. For details on the method see (Rohlf and Slice, 1990) and (Bookstein, 1996).

We ran preliminary analyses for each dataset to identify major outliers that may bias the morphometric analyses by overemphasizing particular directions in the morphospace (and associated morphological characteristics). Such distortion may severely impede sound interpretation of follow-up statistical analyses.

### 20 2.3.2 Statistics

In order to study the covariance between shape variation and environmental variables, two-block partial least-squares analyses (PLS) were performed using software PAST 3.148 (Hammer et al., 2001). As a great advantage over other ordination methods such as principal components analysis, this method disregards within-block variation that may mask between-block covariance (Mitteroecker and Bookstein, 2008, 2011, 2008). Using all relative warps RWs in the PLS might severely bias the pattern, because – contrary to their descending significance in terms of explaining shape variation – they would be treated equally by the analysis. Therefore, we restricted the morphological block to RW 1–20, which account for at least 98.6% of the total shape variation in all four datasets. The environmental variables were log10-transformed to constrain the orders of magnitude involved. PLS analyses was were computed based on the covariance-correlation matrices.

The PLS analysis provides an idea of the overall strength of the relationships with between shape and environment. To detect identify which the parameter(s) contribute to shape variation that affect specific morphological traits or combination of traits. multiple regression analyses were conducted on selected relative warps RWs in the statistical environment R v. 3.3.2 (R Core

Team, 2016). Only warps 1) along which biogeographic differentiation was observed, 2) with an amount of shape variation higher than 10% of the total variation, and 3) [with PLS loading values higher than the mean loading value \(based on absolute values\)](#)~~those yielding high loading values in the PLS analysis~~ were considered. These selection criteria were chosen in order to prevent from misinterpreting seemingly strong relationships between shape and environmental variables. Since the environmental parameters are likely to be highly correlated, eventual [regression](#) models including all variables might be strongly skewed and susceptible to misinterpretation. Therefore, we employed a stepwise selection of variables based on the variance inflation factor (VIF), which is an estimator of multicollinearity among variables (Quinn and Keough, 2002). As a rule of thumb, VIF values greater than ten indicate the presence of multicollinearity (Quinn and Keough, 2002); some authors even consider values above five evidence of collinearity (Heiberger and Holland, 2004). The applied function iteratively removes collinear variables by calculating the VIF of variables against each other (for the script, see [Ijaz, 2013](#)); R package 'fmsb' v. 0.5.2 (Nakazawa, 2015) is required for this procedure. VIF values were calculated with package 'HH' v. 3.1-32 (Heiberger, 2016). To enhance the models further, multiple regressions using backward stepwise selection by evaluation of the Akaike Information Criterion (AIC) were performed with the remaining set of factors.

Normality of model residuals was tested with Shapiro-Wilk tests. In case normality was not achieved, residual distributions were assessed qualitatively using Q-Q-plots; only if the majority of cases match the expected distribution, a model was considered significant. Finally, we used the R package 'hier.part' v. 1.0-4 (Walsh and Mac Nally, 2013) to evaluate the independent contribution of each predictor to the (reduced) models.

### 3 Results

The relative warps analysis ([RWA](#)) yielded different results for males and females, while patterns were largely consistent within sexes (Fig. 2, 3). Along the first three relative warps (~~RW~~), Mexican females have little overlap with Brazilian/Floridian ones. Only some of the specimens from Punta Laguna in northern Yucatan seem to be morphologically closer to the Floridian group and cluster apart in the analyses of both valves. Brazilian and Floridian individuals have a distinctly higher overlap and differentiate only little along RW 2. A clear differentiation within both clusters, like in the Mexican group, is lacking. Group differentiation in male valves is quite contrary: Floridian specimens have little overlap with Brazilian ones in both valves along RW 1, while Mexican specimens are hardly separable from either group along any of the first 3 RWs. However, the differentiation between some Punta Laguna valves and remaining Mexican carapaces along RW 1 is comparable to the patterns observed for females. Mexican and Brazilian males show slight biogeographic differentiation along RW 2 (left valves) and RW 3 (right valves), respectively. No clustering is observed for higher warps in either sex or valve.

Similar to the patterns posed by the scatter plots, [the thin-plate splines indicate that](#) shape variation along RWs is largely consistent within valves but differs slightly between sexes. (Here we ~~display-discuss~~ only axes along which biogeographic discrimination is observed. See Wrozyńska et al., 2016 for within-group variation.) The most important morphological characteristic representing shape ~~change-differences~~ along RW 1 in both females and males and right and left valves, is relative



carapace length (Fig. 2, 3). However, the exact expression differs between sexes: valve outline in males varies between elongate-elliptical and short-asymmetrical with slightly inflated anterior part, and between elongate-elliptical and short-asymmetrical with distinctly inflated posterior region (i.e., brood pouch) in females. In addition to outline differences, the position of the anteriormost pore conulus (LM 2) shifts in dorso-ventral direction consistently in both valves and sexes. In females, also the position of the dorsal dip point of the posterior curvature (LM 6) varies in dorso-ventral direction. Shape variation along RW 2 is in females similar as for RW 1 but with a different combination of traits: negative scores correspond to elongate valves with inflated posterior and slightly shifted LM 2 and LM 6 in dorso-ventral direction. In male *Cytheridella*, only left valves show weak biogeographic differentiation along RW 2, representing shape differences from elongate-elliptical to slightly asymmetrical with higher dorsal margin, and the dorsal dip point of the posterior curvature (LM 6) shifted towards posterior. The only differentiation along RW 3 is for male right valves, corresponding mostly to shell elongation and a little to the relative positions of pore conuli. The relative warp scores of all datasets are provided in Supplementary Table 2.

The PLS analyses ~~show clear~~ indicate relationships between morphological and environmental variables, yet with different results for males and females, with similar results across both valves and sexes. The first PLS axis explains between 68.7% and 77.9% of the total variation, whereas values are consistently higher for females (LV: 77.5%; RV: 77.9%) than for males (LV: 68.7%; RV: 71.5%). In all four analyses, Brazilian specimens are widely separated from Floridian/Mexican ones along PLS axis 1, corresponding to a clear differentiation along both environmental and morphological scores. Left valves of females and left and right valves of males of Brazilian specimens exhibit negative scores on both PLS axes corresponding to shape and environmental variables. Females display inverse distributions for Brazilian and Mexican specimens. Floridian and Mexican groups overlap little but consistently in all analyses, while the specimens of Florida tend to have smaller variation ranges than Mexican groups (Fig. 4). Permutation tests indicate however that PLS analyses are hardly significant for male valves (LV: P = 0.126, RV: P = 0.135). Yet, the low significance levels do not necessarily imply lacking relationships between shape and environment, they rather witness the difficulties in finding clear relationships in multifactorial analyses. In fact, the overall picture provided by the PLS might mask individual relationships between selected shape traits and environmental parameters, which is why a closer look is required by using multiple regressions. Nonetheless, the PLS analysis are useful to examine the overall strength of the relationships, which seem to be stronger and clearer in females than in males.

For PLS axis 2, low relationships between shape and environment are yielded for all four datasets, and none of them are significant (see Supplementary Table 433).

The loadings for morphological variables in the PLS analyses yield constantly high values for RW 1; RW 2 shows loading values higher than the mean (based on absolute values) in ~~is important to~~ all analyses except male right valves; RW 3, in turn, contributes above average to variation in all cases but females right valves in male valves but hardly in females. Higher-Other warps were not considered because of their minor influence on shape variation (low loading values) or the lack of biogeographic separation. See Table 1 for a summary of the results.

Following warps fulfil the selection criteria defined in the M methods section for consideration in the multiple regressions: RW 1 for all four datasets; RW 2 for female right and left valves ~~and male left valves~~; RW 3 for female left valves and male right

and left valves. Hence, nine regression analyses were carried out. Shapiro-Wilks tests of model residuals indicate normality for ~~four of the nine analyses of males but not females~~ (Table 12). Inspection of Q-Q-plots yielded, however, that in all models the majority of cases match the expected distributions, which is why the ~~remaining~~ models ~~for females~~ are still considered significant (see ~~Supplementary Figure 1 Appendix S2~~). ~~Seven-Eight~~ out of nine models are significant ( $P < 0.05$ ); the models

5 for ~~RW 2 and~~ RW 3 for male left valves ~~are is~~ not ( $P = 0.08$  and  $P = 0.074$ , respectively).

Only a limited set of predictor variables is retained out of the originally 15 variables in each model ~~after elimination of collinear parameters and backward stepwise selection~~ (see ~~Supplementary Table 4~~). Seven parameters do not contribute to any models:  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{HCO}_3^-$ , ~~and~~ conductivity, mean annual temperature, and precipitation seasonality. Of the remaining factors, temperature seasonality is one of the most important predictors in almost all models, accounting for at least 28.7% in all models

10 with RW 1. Temperature seasonality is highest in Florida, closely followed by Brazil, and considerably less in Mexico, reflecting the distinction between Mexican and Floridian/Brazilian populations along RW 1. Similarly, annual precipitation and the anions  $\text{Cl}^-$  and  $\text{SO}_4^{2-}$  contribute significantly to many models, corresponding to differences in the hydrological regimes. Less explanation power ~~is provided~~ ~~s by~~ pH,  $\text{K}^+$ , water temperature, and mean diurnal temperature range. It is noteworthy that anions, represented by  $\text{Cl}^-$  and  $\text{SO}_4^{2-}$ , are obviously much more important than cations.

## 15 4 Discussion

Variation in temperature seasonality, annual precipitation and anions ( $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ ) explain a large portion of shape variation in *Cytheridella*, which is mostly related to relative carapace length and outline shape. Narrow elongate shapes, such as those occurring in Mexico, correspond to relatively low seasonality and precipitation but high anion concentrations. Opposite conditions ~~seem to~~ favor the formation of short, asymmetrical valves typical for specimens from Florida and Brazil. Secondary

20 shape variations differentiating between elongated valves with slightly wider posterior and short, symmetrical valves (i.e., RW2) are attributed to higher and lower annual precipitation, respectively.

The link between shape variation and environmental conditions is a well-studied branch of ostracodology, but studies have yielded quite contrasting results. Frequently identified ecological factors are salinity (Yin et al., 1999; Yin et al., 2001; Grossi et al., 2017) and hydrochemical regime, mirrored by  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{K}^+$  contents (Ramos et al., 2017) or alkalinity and sulphate,

25 respectively (van der Meeren et al., 2010). Morphological response to the same environmental factor may even differ between environments (e.g., Yin et al., 1999), complicating straightforward explanation models.

### 4.1 Potential environmental drivers of valve shape variation

The mechanisms that control the relationship between valve shape and environmental gradients in *Cytheridella* are not understood at present. Like many non-marine ostracods, *Cytheridella* is characterized by a benthic life style. Growth and proliferation of an individual and a population, respectively, might benefit from changes in carapace shape with respect to different habitats. For instance, a more elongated shape could be advantageous in more densely vegetated environments

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because of increased motility. For the present study, we sampled various habitats within each region, differing in vegetation cover and composition (Supplementary Table 1). If shape differences were indeed functional adaptations to varying habitat conditions, we would expect much higher morphological variability within each region and smaller differences between specimens from similar habitat types than shown by the analyses. We rather suppose that shape difference in *Cytheridella*

5 *ilosvayi* has a physiological origin that mirrors the varying environmental conditions.

The geographical range of *Cytheridella* coincides with the Neotropical region, which spans a wide latitudinal range from ~30°N to ~30°S. This range involves a latitudinal decline in mean annual temperature, which mainly corresponds to differences in annual minimum temperature (Lewis, 1996). ~~Both~~ Florida and southern Brazil are characterized by higher annual temperature gradients compared to Mexico. Annual minimum and maximum temperatures ~~in Florida and southern S Brazil~~ range between 16°C and 30°C ~~in Florida~~ and 10°C and 30°C ~~in southern Brazil~~, respectively. Minimum and maximum temperatures vary between 19°C and 33°C (Climate-Data.org, 2017).

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Temperature has a direct effect on other environmental parameters such as salinity and oxygenation of the water. Water temperature is one of the most important variables affecting metabolism, oxygen consumption, growth, molting and survival of crustaceans (Le Moullac and Haffner, 2000 and references therein). Increases in temperature can result in significantly shortened intermolt periods, higher molting rates (Roca and Wansard, 1997; Mezquita et al., 1999; Brylawski and Miller, 2006), increased growth increments (Martens, 1985; Iguchi and Ikeda, 2004) and reduction in maturation time (Pöckl, 1992).

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We expect that higher temperature seasonality induced prolonged molt cycles in populations of *C. ilosvayi* by extending intermolt periods during colder seasons. ~~We hypothesize that How these changes affected valve shape is, however, not understood at present, the changed molt cycles affected calcification patterns and led to the observed differences in shape. Since~~ the physiological processes involved in the secretion of ostracod valves are poorly known (and not at all for *Cytheridella*), this hypothesis cannot be tested at present.

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Precipitation causes declines in nutrients and promotes physical disturbance of the water column (Figueredo and Giani, 2009). Moreover, changes in precipitation directly influence hydrochemical composition, input of sediments, organic components and contaminants and lake level (Mortsch and Quinn, 1996; Whitehead et al., 2009). Indirect influence ~~represent~~poses, e.g.,

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the control on aquatic plants, which represent important (micro)habitats and/or food sources (Lacoul and Freedman, 2006). The annual cycle of precipitation over most of South America is monsoon-like with great contrasts between winter and summer (Grimm et al., 2007). ~~The peak rainy season in the Brazilian sample region is the austral winter. The rainfall is caused by frontal penetration associated with migratory extratropical cyclones In the region the Brazilian samples derive from, the peak rainy season is the austral winter, a mid latitude regime, where the rainfall is due to frontal penetration associated with~~

30 ~~migratory extratropical cyclones~~ (Grimm et al., 1998). The amount of rainfall in Yucatan is associated with seasonal migration of the Intertropical Convergence Zone and less by spatially oriented tropical convective activity (e.g., Hodell et al., 2008).

Florida, in particular Southern Florida, where most of ~~the our~~ sampled areas derive from, receives maximum precipitation during northern hemisphere summer from convectional and tropical storms (Schmidt et al., 2001). The annual precipitation amounts for the sampled areas are with 1396–1492 mm per year in Brazil higher on average than in Florida and Yucatan, with

1185–1430 mm and 1125–1359 mm, respectively. Since the annual amounts of the regions are very similar it might be more plausible that precipitation seasonality has an influence on carapace shape of *Cytheridella* through seasonally restricted nutrient inputs or changes of the hydrochemistry. Annual precipitation should be therefore considered with caution since it is difficult to deduce a causal relationship with carapace shape.

5 Ionic composition of the host water is vital for calcification and growth rates of ostracods (Mezquita et al., 1999). The relationship between hydrochemistry and phenotypic variability is poorly understood, however. A ~~latest~~ study of Kim et al. (~~Kim et al., 2015~~) shows that increased levels of pH account for decreased carapace growth rates, i.e., prolonged intermolt periods, and smaller carapaces. Carapace shape ~~changes-differences~~ have been moreover associated with changes in  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and pH (Ramos et al., 2017). Our analyses, however, revealed correlations neither with ions related to formation of carbonate, such as  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$ , nor with pH. Only chloride and sulfate contents significantly ~~explain-correlate with~~ carapace shape variation.

Natural sources of  $\text{Cl}^-$  in freshwater derive from marine sprays transferring NaCl into the atmosphere and are ~~either~~ transported as aerosol by ~~wind, and wind or are~~ washed out by precipitation and the weathering of rocks. ~~Additionally~~~~Additionally~~, large amounts of chloride are derived anthropogenically from farming and waste water production (Müller and Gächter, 2012).

15 Sulfate can derive through runoff from mining and agricultural areas, mobilization from pyrite deposits by oxygen intrusion during desiccation and weathering of rocks containing ~~Sulphur-sulphur~~ (Holmer and Storkholm, 2001; Lamers et al., 2002). Sulfate contents in groundwaters and surface waters result from dissolution of gypsum and anhydrite occurrences (Perry et al., 2002) and ~~from~~ mixing with seawater (Sacks et al., 1995). In Yucatan, a gypsum-rich stratigraphic unit occurs providing a solution-enhanced subsurface drainage pathway for a broad region extending along the eastern coast and from east to west in  
20 the southern part (Perry et al., 2009). The chloride content of groundwater is the result of mixing with seawater (Mondal et al., 2010). Additionally, a  $\text{Cl}^-$  gradient extends from southeast to northwest providing generally higher chloride contents (Perry et al., 2009). Concentration gradients of  $\text{SO}_4^{2-}$  and  $\text{Cl}^-$  in Florida occur from inland to coastal areas as well as with depth (Sacks et al., 1995), explaining the relatively higher amount of chloride and sulfate. The comparably low values for south Brazilian sampling locations is not surprising given that such coastal water bodies are often fed by groundwater (Santos et al., 2008) that  
25 ~~are-is~~ dominated by bicarbonate waters and low chloride and sulfate contents (Gianesella-Galvão and Arcifa, 1988; Viero et al., 2009). The detected relationship between morphotypes and chloride and sulfate contents, respectively, could thus mirror the hydrochemical compositions resulting from different hydrogeological conditions of the regions.

~~(Van der Meeren et al., 2010)~~ found ostracod valve shape variability to be significantly correlated with the ratio between alkalinity and sulfate. As the ratio was inversely related to solute concentration, the authors hypothesized that carapace shape  
30 may be linked to changes in the lake water balance or relative climatic moisture, or changes in the sources of solutes delivered to the environment. Varying anionic composition has also been considered to affect osmoregulation and calcification (Mezquita et al., 1999). As hyperosmotic organisms, freshwater ostracods are obliged to pump ions inwards (mainly  $\text{Na}^+$  and  $\text{Cl}^-$ ) and water outwards to maintain a stable internal ionic concentration higher than that of the ambient water (Weihrauch et al., 2004). Chloride is obtained from the environment through a  $\text{HCO}_3^-/\text{Cl}^-$  antiport pump. The organism needs to precipitate calcite but

also pump  $\text{HCO}_3^-$  outwards to maintain the internal  $\text{Cl}^-$  concentration (Mezquita et al., 1999). These authors assumed that even small genetic differences affect varied ecophysiological responses to temperature and water chemistry, which may be a key factor for the explanation of different biogeographical patterns of non-marine ostracods. Especially the trade-off between ionic regulation and calcification is considered to play a key role in ostracod speciation (Mezquita et al., 1999).

- 5 ~~One of the best-studied phenomenon~~phenomenon in ostracods is (variable) nodding (hollow outward flexions on the lateral surfaces on the valves) of *Cyprideis* (e.g., Vesper, 1975; van Harten, 2000). A connection between node formation and salinity was noted early, but the reported salinity limits are partly contradictory (Keyser and Aladin, 2004). Frenzel et al. (2012) deduced from a combination of mesocosm cultures and field studies that nodding of *Cyprideis torosa* valves is pathologically and caused by osmotic problems under lower salinities and lacking  $\text{Ca}^{2+}$  during molting. From the same species it is known
- 10 ~~that increasing salinity corresponds with decreasing proportion of rounded sieve pores of the valves (Frenzel et al., 2017). The role of sieve pores in osmoregulation or, the reason for their shape change is still not clear. If~~Considering that the variability of discrete valve traits such as nodding or sieve pore shape is caused by~~related to complex physiological processes, we hypothesize that the relationship between the same might applies for~~carapace shape and ionic composition detected by our analyses could be~~is a result of osmoregulation~~complex interplay of different physiological processes affecting valve calcification. Understanding the physiological processes involved requires more detailed studies. Not to forget that it might be
- 15 ~~species-specific. This requires combined investigations using mesocosm experiments and field observations.~~

#### 4.2 Genetic diversity or ecophenotypic plasticity?

- 20 Phenotypic variation in ostracods is considered to reflect either genotypic or ecophenotypic variability or a combination of both (Martens et al., 1998; Yin et al., 1999; Anadón et al., 2002; Frenzel and Boomer, 2005; Boomer et al., 2017; Grossi et al., 2017). A recent study on valve outline variability of a non-marine ostracod demonstrated that differences in carapace shape do not correspond to genetic clades (Koenders et al., 2016). However, caution is advised when comparing patterns among species, since different species react differently and have different potentials for ecophenotypic variation (Anadón et al., 2002;
- 25 Frenzel and Boomer, 2005). ~~Nonetheless, the here documented morphological differences in carapace shape among populations from Mexico, Florida and Brazil, which are consistent to patterns shown for appendages (compare Wrozyrna et al., under review), may argue for genetic differentiation. This hypothesis is supported by the fact that soft parts are assumed to be more conservative to environmental influences (Park et al., 2002; Park and Ricketts, 2003). Similarly to (Koenders et al., 2016), we therefore assume that the observed morphological cluster correspond to cryptic species. Wrozyrna et al. (submitted)~~
- 30 ~~tentatively ascribes the currently widespread distribution of the “*Cytheridella ilosvayi* species group” to passive dispersal via birds. Local populations are considered to have adapted to prevailing environmental conditions, followed by morphological and, eventually, genetic divergence. This highly speculative scenario needs to be tested using molecular data, which are not available at present however.~~

The relationship between genotype and environment might differ among species, geographical regions and through time (see, e.g., Sanchez-Gonzalez et al., 2004; Koenders et al., 2016). ~~Concluding from~~ Our statistical results it is clearly implied that morphological disparity ~~of~~ in *Cytheridella* is controlled by environmental factors. However, the distribution and the variation range of regional clusters reveal some opposing implications. For instance, a part of the Mexican populations group comprises specimens with similarly (shortened) valves ~~shapes~~ as are found in the Floridian group. Both shortened and ~~but co-occur with~~ the elongated morphotypes co-occur in one lake (i.e., Punta Laguna) (Wrozyńska et al., under review). On the one hand, this co-occurrence could suggest the presence of microhabitats with specific environmental conditions, posing differential impact on valve calcification in the very same ecosystem. On the other hand, this discrepancy might be considered evidence for genetic differentiation. An integrated study combining genetic and morphometric data is required to further explore this case.

~~Since morphological variability of the appendages reflects, the pattern revealed by hard parts it is assumed that regional morphotypes of *Cytheridella* represent (cryptic) morphospecies. Wrozyńska et al. (Wrozyńska et al., submitted) provide a hypothesis that widespread distribution of *Cytheridella* may have originated through, e.g., passive dispersal and how local populations may have adapted to local environmental conditions accompanied by successive morphological divergence and eventually speciation.~~

~~A higher morphological similarity between Florida and Brazil can be explained by interconnection of these regions through, e.g., avian dispersal. Ecological conditions in Mexico (e.g., higher or more variable salinity) could have been promoted the evolution of new species (with more elongated outlines) which have occupied all available niches preventing colonization through other species. This model allows to explain the co-occurrence of two different morphotypes (species) in one lake. It cannot be ruled out that temperature seasonality and chloride and sulfate concentrations have contributed to these processes.~~

## 20 5 Conclusion

The comparison of our results and a large number of previous studies witnesses the difficult nature of ecophenotypic response to varying climatic and ecological conditions in freshwater ostracods. Shape variation in *Cytheridella*, mostly related to relative carapace length and outline shape, is mainly explained by temperature seasonality, annual precipitation and chloride and sulfate compositions. Increased temperature seasonality, characteristic for Florida and south Brazil, are considered to account for slower growth rates during colder months and may have triggered the development of shortened valves with well-developed brood pouches. We propose that ~~D~~ differences in chloride and sulfate concentrations, which are ~~also~~ related to fluctuations in precipitation, might have affected valve development via controlling osmoregulation and carapace calcification. These explanation models are, however, tentative as physiological studies on the influence of changing ecological conditions in non-marine ostracods are still scanty. This ~~A more detailed picture will require~~ requires combined investigations using mesocosm experiments and field observations.

Temperature *per se*, salinity (expressed as electrical conductivity) and pH have surprisingly little or no effect on shape variation in *C. ilosvayi*, although these factors have been discussed as important drivers of ostracod ecophenotypy, variably affecting

size, ornamentation and shape. The discrepancies in explanation models suggest that environmental predictors for valve shape are not consistent across non-marine ostracods. The nature of the phenotype–environment relationship likely depends on the choice of the model taxon and ecosystem. On a larger scale, this lack of a general pattern complicates reconstruction of paleoenvironments based on ecophenotypic variation.

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#### **Data availability**

All relevant data are presented within the manuscript or in supplementary material.

#### **Supplement link**

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#### **Author contribution**

C. Wrozyzna, J. Meyer and W. E. Piller carried out sampling of ~~modern~~ *Cytheridella* populations. C. Wrozyzna and J. Meyer prepared ostracod material for morphometric analyses. T. A. Neubauer performed statistical analyses. ~~Preparation of the~~ manuscript was ~~done-written~~ by C. Wrozyzna and T. A. Neubauer, ~~and~~ M. I. F. Ramos contributed to the discussion of the results and provided taxonomic advice.

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#### **Competing interests**

The authors declare that they have no conflict of interest.

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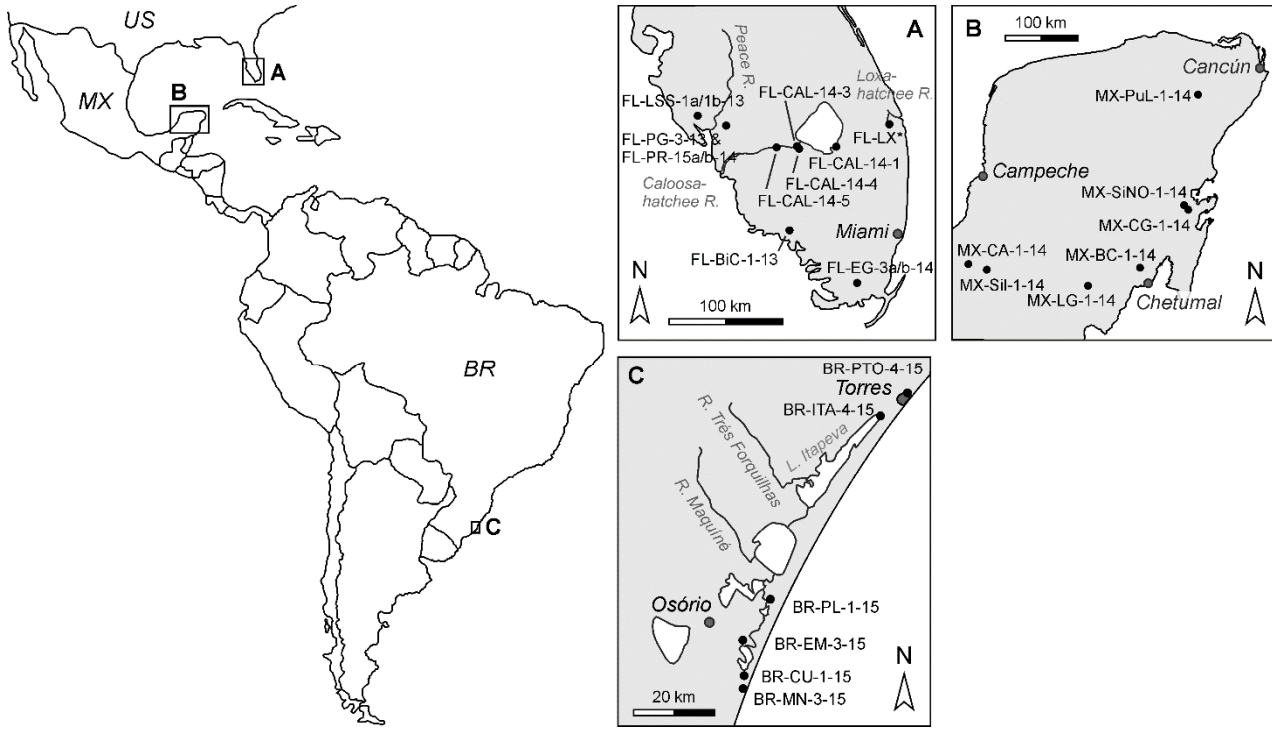
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5 **Figure 1: Geographic overview of the sampled populations (modified from Wrozyńska et al., 2016). The label FL-LX\* in map A comprises samples FL-LX-1-14 to FL-LX-6-14. For details, see Table S1.**

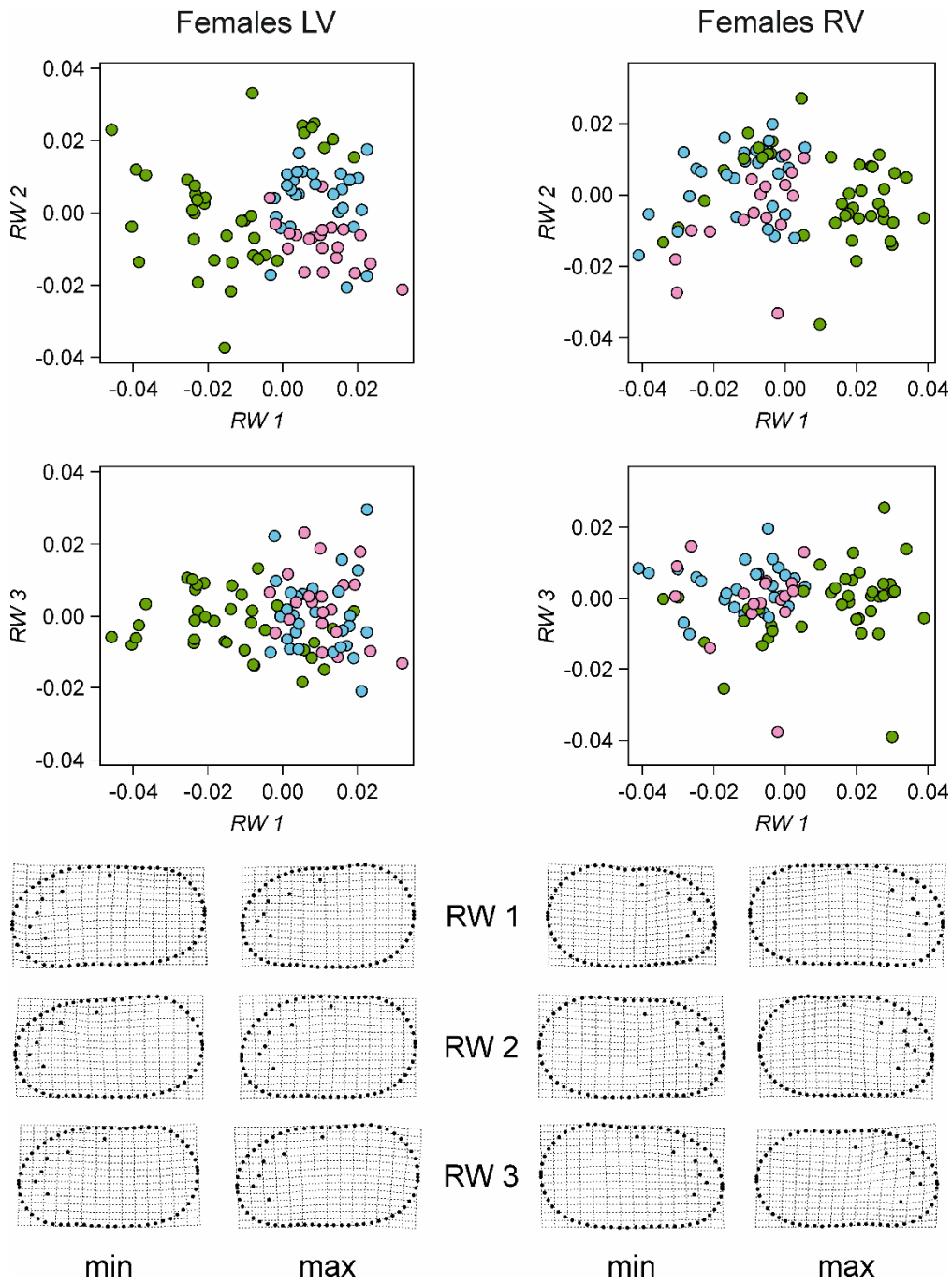


Figure 2: Relative Warps Analyses of left and right valves of females of the first three warps and the associated thin-plate splines at minimum and maximum scores. Colors refer to the different regions: blue – Florida, green – Mexico, pink – Brazil.

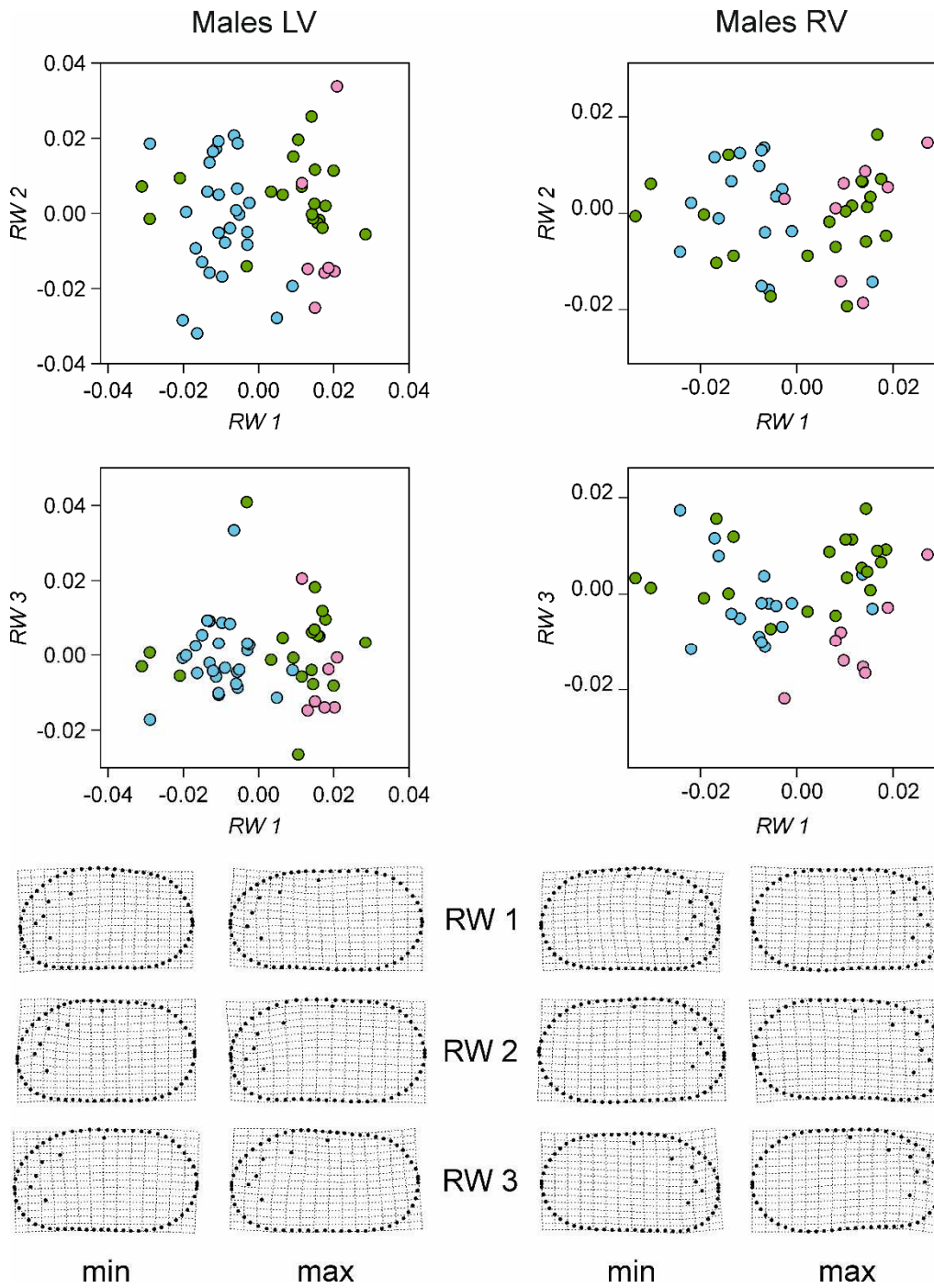


Figure 3: Relative Warps Analyses of left and right valves of males of the first three warps and the associated thin-plate splines at minimum and maximum scores. Colors refer to the different regions: blue – Florida, green – Mexico, pink – Brazil.

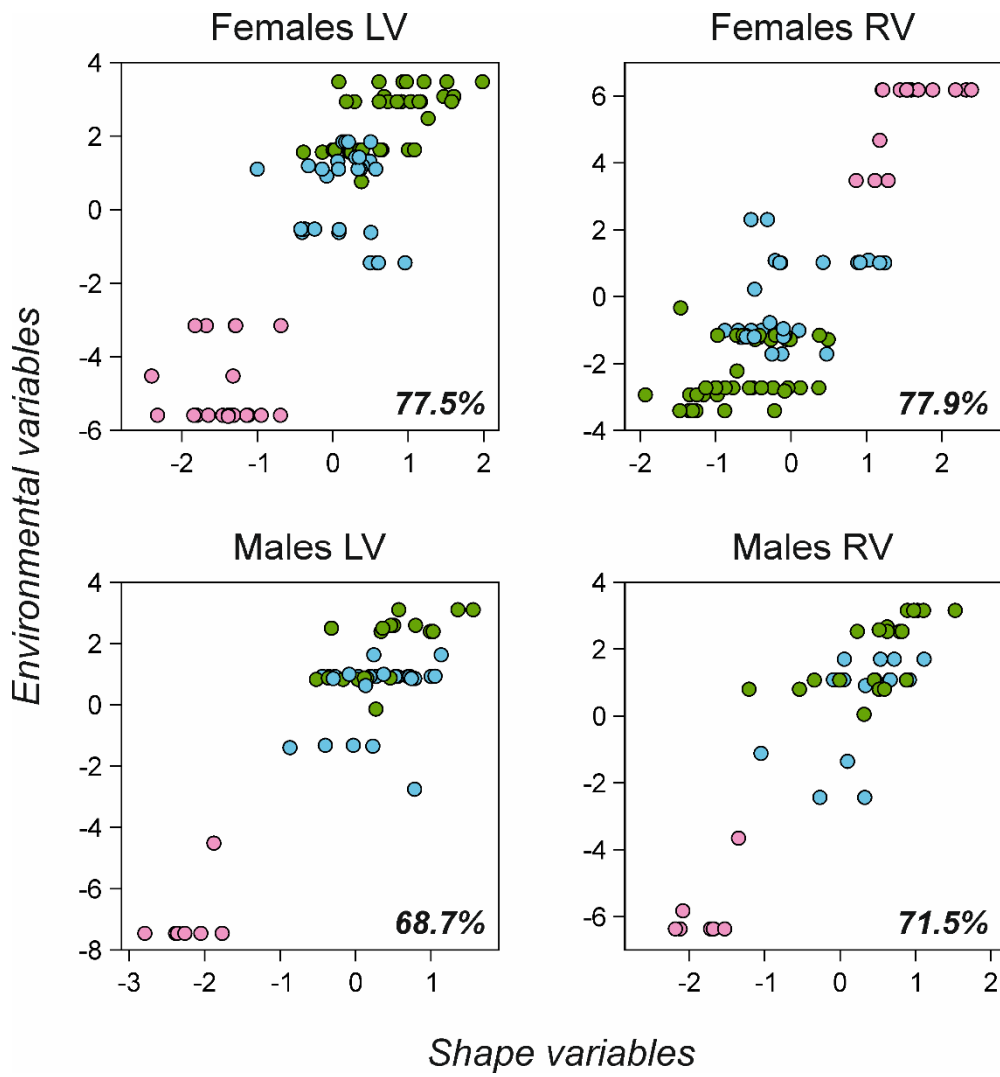


Figure 4: First axis of the Partial-least-squares-PLS analysis of carapace shape and environmental variables. Colors refer to the different regions: blue – Florida, green – Mexico, pink – Brazil.



**Table 1. Summary statistics Results of the RWA, PLS analyses and multiple regression analyses for RWs 1–3 for the four datasets. The reification column summarizes shape variation along the respective warp.- For the multiple regressions, percentage values are indicated for predictor variables with relative contributions over 10 %. Bio2-BIO2 – Mean diurnal temperature range (mean of monthly (max – min temperature)), Bio4-BIO4 – Temperature seasonality (standard deviation \* 100), Bio12-BIO12 – Annual precipitation. See Methods chapter for details.**

<i>Females LV</i>	<i>RWA</i>		<i>PLS</i>	<i>Multiple Regressions</i>			
	% <u>var. explained</u>	<u>Reification</u>	<u>Loadings axis 1 (77.55%)</u>	<u>R<sup>2</sup><sub>adj</sub></u>	<u>P</u>	<u>Shapiro-Wilks test (P)</u>	<u>Predictor variables</u>
RW_1	33.22	<a href="#">elongate-elliptical vs. short asymmetrical with distinctly inflated posterior region; position of pore conuli</a>	-0.5637	0.632	<0.001	0.0200	BIO4 (46.2%), SO <sub>4</sub> (19.3%), pH (13.0%), Cl <sup>-</sup> , Temp., BIO12
RW_2	19.00	<a href="#">elongate-elliptical with slightly inflated posterior region vs. short; position of pore conuli</a>	0.4798	0.510	<0.001	0.0063	BIO12 (43.9%), Cl <sup>-</sup> (32.9%), K (11.7%), SO <sub>4</sub> , BIO4
<u>RW3</u>	<u>10.72</u>		<u>-0.1850</u>	<u>n/a</u>			
<u>RW 3</u>	<u>10.72</u>	<u>minor deviations of outline; small differences in position of pore conuli</u>	<u>-0.1850</u>	<u>0.142</u>	<u>0.002</u>	<u>0.3338</u>	<u>SO<sub>4</sub> (33.3%), Temp. (27.5%), Cl<sup>-</sup> (24.6%), BIO4 (14.6%)</u>
<i>Females RV</i>	<i>RWA</i>		<i>PLS</i>	<i>Multiple Regressions</i>			
	% <u>var. explained</u>	<u>Reification</u>	<u>Loadings axis 1 (77.95%)</u>	<u>R<sup>2</sup><sub>adj</sub></u>	<u>P</u>	<u>Shapiro-Wilks test (P)</u>	<u>Predictor variables</u>
RW_1	40.39	<a href="#">elongate-elliptical vs. short asymmetrical with distinctly inflated posterior region; position of dorsal dip point of the posterior curvature; position of pore conuli</a>	-0.3892	0.593	<0.001	0.0079	BIO4 (44.5%), Cl <sup>-</sup> (20.4%), SO <sub>4</sub> (18.1%), Temp., K, BIO12
RW_2	14.89	<a href="#">elongate with slightly inflated posterior region vs. short; position of dorsal dip point of the posterior curvature; position of pore conuli</a>	-0.2373	0.257	<0.001	0.0040	BIO12 (48.4%), SO <sub>4</sub> (23.5%), Cl <sup>-</sup> (20.1%), BIO4
RW_3	10.50	<a href="#">elongate-elliptical vs. short asymmetrical with inflated posterior region; position of dorsal dip point of</a>	0.1003	<u>n/a</u>			

		<a href="#">the posterior curvature; position of pore conuli</a>					
<b>Males LV</b>	<b>RWA</b>		<b>PLS</b>	<b>Multiple Regressions</b>			
	<b>% var. explained</b>	<b>Reification</b>	<b>Loadings axis 1 (67.74%)</b>	<b>R<sup>2</sup><sub>adj</sub></b>	<b>P</b>	<b>Shapiro-Wilks test (P)</b>	<b>Predictor variables</b>
RW_1	24.14	<a href="#">elongate-elliptical vs. short asymmetrical with slightly inflated anterior part; position of pore conuli</a>	-0.4063	0.638	<0.001	0.3948	BIO4 (37.3%), Cl <sup>-</sup> (34.5%), BIO12 (15.0%), SO <sub>4</sub> (10.8%), pH
RW_2	22.25	<a href="#">elongate vs. short with higher dorsal margin; position of pore conuli</a>	0.2359	<i>n/a</i> 0.058 0.082 0.4130 (not significant)			
RW_3	13.92	<a href="#">minor deviations of outline; position of pore conuli</a>	0.2792	0.042	0.074	0.0017	(not significant)
<b>Males RV</b>	<b>RWA</b>		<b>PLS</b>	<b>Multiple Regressions</b>			
	<b>% var. explained</b>	<b>Reification</b>	<b>Loadings axis 1 (71.55%)</b>	<b>R<sup>2</sup><sub>adj</sub></b>	<b>P</b>	<b>Shapiro-Wilks test (P)</b>	<b>Predictor variables</b>
RW_1	29.88	<a href="#">elongate-elliptical vs. short- asymmetrical with slightly inflated anterior part; position of pore conuli</a>	-0.3798	0.318	0.001	0.2221	BIO12 (31.8%), BIO4 (28.7%), Cl <sup>-</sup> (15.8%), pH (12.5%), K (11.2%)
RW_2	12.81	<a href="#">elongate vs. short with higher dorsal margin; position of dorsal dip point of the posterior curvature; position of pore conuli</a>	0.0157	<i>n/a</i>			
RW_3	11.98	<a href="#">shape of anterior part; position of pore conuli</a>	0.5502	0.286	<0.001	0.2978	BIO4 (57.2%), BIO2 (42.8%)