

Dear Editor,

I am sending you our revised manuscript “Impact of climate and hydrochemistry on shape variation – a case study on Neotropical cytheroidean Ostracoda” and replies to the referee comments.

5 The repeated critics of referee #1 are related to the statistical approach and the request to provide explanations how environment impacts on the carapace shape. We want to emphasize here once more that the statistical approach using PLS analyses was chosen to test for the overall covariation between morphology and environment. Because of the complexity of environment and shape, which the referee is explicitly referring to, we used multiple regressions that allow a more precise estimation of the parameter affecting specific shape traits. It is the combined
10 power of PLS and multiple regressions that help to disentangle the relationships of shape and extrinsic factors.

We cannot follow the referee’s comment that explanations for how environmental changes influence shape changes are important for considering acceptance for publication. Despite the lacking physiological explanations (which are simply not possible from our data), our study is the first that characterizes the relationship of environmental variables and associated morphological changes on a large geographical scale. Moreover, we do discuss the results
15 as broadly as possible. We believe that our study provides a useful baseline for further, complementary studies with more specific research objectives such as the physiological processes involved in morphological changes.

Referee #3 seemed to be uncertain about the novelty of the study. In order to clarify that point, we have emphasized in the MS that the dataset covering a supra-regional scale and hydrochemical and climatic data represent a novel contribution to research on ecophenotypy.

20 Our study provides new insights and thought-provoking impulses in terms of species–environment interactions and can be important for interpreting potential future studies. As such we think this manuscript is of interest not only to specialists on ostracods or freshwater ecologists but to a broad readership of geoscientists and biologists in general.

Please find more in-depth replies to the referees’ comments attached.

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Yours sincerely,
Claudia Wrozyńska

Reply to Anonymous Referee #1 (Report submitted on 13 June 2018)

5 We thank the referee for his/her constructive comments on the revised manuscript.
His/her major critics refer to the inferences of the PLS analyses and non-reasonable explanations for how the environment impacts shape variation in *Cytheridella*.

10 “I have checked the revised manuscript, but the revised manuscript does not reply or refute to some of my concerns. For example, the places of the specimens from Punta Laguna in the morphospace (Figs. 2 and 3) are not clarified.”

- In our first revision and reply to the referees comments we have carefully checked all of his/her requests. We obviously overlooked this point. We will prepare a revised figure indicating Punta Laguna.

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“1) Because PLS maximizes the correlation of shape variables and environmental variables, I think authors should evaluate the results of PLS carefully. Graphical presentation of shape deformation indicated by PLS singular axis of shape variables should be added. In the first PLS singular axis, there seems to have no excess values of loadings in specific environmental variables. This may be due to the fact that the environment is complex. So simplifying the focus on specific environmental variables may be misleading, despite selection of variables by VIF. Although the similarity between Florida and Brazil is emphasized, the results of PLS analyses seem to indicate the similarity between Florida and Mexico. The discrepancy is not mentioned in the responses.”

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- There seems to be a confusion here. The PLS loading values for the environmental variables are partly quite high and do warrant an interpretation. However, we intentionally did not go into the details for the PLS exactly because of the complexity of environment and shape. In fact, the complete picture presented by the PLS might not be able to show the effect of environmental parameters on specific shape traits. Also, we did not stick to “specific environmental variables” as claimed by the referee; the multiple regressions included several parameters, only we focused on single warps that correspond to specific shape traits. An aim of the paper was to find out which environmental parameters/combination of parameters are responsible for differences in which shape trait(s). This question was answered using multiple regressions and could not be done so only using PLS. Moreover, the software we used for the PLS analyses does not offer the possibility of generating thin-plate splines (but we do not consider this a drawback because the stronger focus in on the regressions).

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- Concerning the similarities of the three regions, the referee seems to have overlooked that we had already responded in our first reply to the apparent “discrepancy” of the similarities. This contradiction is caused by mixing two different aspects. The emphasis of the similarity between Florida and Brazil refers to the morphologies (given in the Relative Warps Analyses), both regions provide rather shortened carapace outlines compared to Mexican carapaces which are strikingly different. The PLS indeed reflects the

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relationship between environmental gradients and shape changes. The higher ‘similarity’ of Florida and Mexico in the PLS is caused by similar environmental factors. For instance, Florida and Mexico cover wider conductivity ranges (205 to 2360 $\mu\text{S/cm}$) whereas Brazil is represented by low conductivities ($\leq 279 \mu\text{S/cm}$). We believe this is clearly stated in the text.

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“In this revision, authors added some explanations, but they are not so persuasive or well-structured for emphasizing “impact of climate and hydrochemistry”. The authors indicate the environmental difference among regions, but do not provide reasonable explanations for how the environment impacts on shape variation. Because the novelty of this manuscript is the relationship between shape and environment (the geographic variation of shape seems to be another study by authors; Wrozyzna et al. 2016, under review), this is important for considering acceptance for publication.”

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- The referee asks for “reasonable” explanations how the environment impacts on shape regions, but it is unclear what kind of discussion he/she wants to see exactly. Since no further comments are provided to explain this, we assume that he/she expects physiological explanations, i.e., environmentally induced processes that lead to shape changes of the carapaces. We fully agree with the referee that this would be highly required especially since it could contribute to understand if the control of environmental parameters on morphology are, e.g., species-specific, related to geographical scale (local populations vs. geographical range of a species or habitat type), interact with other processes and/or parameters, etc. However, this requires complementary physiological studies that are unfortunately unavailable as yet.
 - Nevertheless, we do provide explanation models in the discussion. The key process that combines causes and consequences is the calcification of new valves during molting. Due to the small size of ostracods and the rapidity of the process (usually hours to few days) field studies are not the appropriate approach. Mesocosm experiments, in turn, allow the observation of specimens under controlled hydrochemical conditions. Previous ostracod-related mesocosm experiments have been studied with respect to restricted number of variables with a clear focus on the effects of salinity changes.
 - Our dataset contains not only salinity and other principle hydrochemical variables but also climatic data. This is a major difference to all other previous studies. They are dealing either with mesocosm experiments or with (a restricted number) of field populations and hydrochemical data only. Hydrochemical data can vary on very small spatial scales in contrast to climatic data. Thus, so far only local or regionally-restricted relationships have been considered. While we do not want to lower the relevance of these previous studies, our inter-regional approach is the first ever that investigates environmental impacts on the morphology on a (large) geographical scale that coincides with the geographical range of the investigated species and allows inferences about the environment-shape relationships inter-regionally.
 - In summary, we cannot provide explanations how environment impacts on shape in terms of physiological processes, but we think that our study provides new insights and thought-provoking impulses in terms of species-environment interactions and can be important for interpreting potential
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future studies. Nonetheless, we extended the discussion to emphasize the novelty of the study in order to meet the referees' critics that explanations are not persuasive and well-structured.

- In order to follow the referees' concerns that the title/statement of the manuscript is not justified we change it into: "Significance of climate and hydrochemistry ...".

Reply to Anonymous Referee #3 (Report submitted on 09 May 2018)

We thank the referee for his/her constructive comments on the revised manuscript.

5 He/she formulates critics on the missing emphasis on the novelty of the study.

“Authors revised the MS accordance with referee’s comments. Especially, to explain the mechanisms that can change the valve shape, authors revised MS based on a lot of previous studies. However, authors also had to emphasize the novelty of study, in this revised version. Authors introduce a lot of previous studies that show relationships between ecophenotypic variation of freshwater ostracods and environmental variables. This is fine but the value of this study become ambiguous.”

➤ The introduction of previous studies is necessary in order to show inconsistencies in the approaches to study ecophenotypical characteristics as well as the conclusions made from the different studies.

Furthermore, this provides the motivation for the present manuscript and its novel approach.

15 Nevertheless, we added a sentence at the end of the introduction to explicitly highlight the novelty of the study.

“In addition, true, valve shape seems to be influenced by some environmental variables but the effect of random genetic drift was not excluded. Authors should discuss about it.”

20 ➤ We agree with the reviewer that genetic drift must be considered for the interpretation of morphological variation. Indeed, we had the intention to characterize the genetic divergence of the different morphotypes. However, due to unknown reasons these analyses failed (B. Stelbrink, University of Giessen, personal communication). In order to avoid extensive discussion that cannot be tested by our data, we restricted the discussion to the comment that genetic differentiation might be an explanation for some discrepancies such as the co-occurrence of morphotypes in one locality. Any further discussion would be highly speculative.

Minor comments

30 ▪ I commented you should introduce life cycle of Cytheridella. Not every reader is specialist about Cytheridella, especially in journals like BGS that accept variety field studies. Current MS is a little hard to understand the significance of this study, if he/she don’t know ecological information, such as life span and dispersal ability that affect morphological evolution.

35 ➤ We totally agree with the reviewer that the manuscript would benefit from such important information. This was already requested by one of the previous reviewers. Until then, information about life cycle, life

span and reproduction were not available for *Cytheridella* in the literature and cannot be deduced from our data. However, a recently published paper of our working group provides now more information on life span, molt cycles and calcification periods of *Cytheridella*. We will add that information in the revised discussion.

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▪ Although paper writing style differ from person to person, I think the word “hypothesize” should not be used in discussion part. Of course, I know this revision was based on another referee’s comment. But I think authors should change this description. For example, “Our results suggest _____ because some previous studies showed _____”.

➤ We changed the phrases according to reviewer suggestions.

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List of changes

➤ In order to follow the referees’ concerns that the title/statement of the manuscript is not justified we change it into: “Significance of climate and hydrochemistry ...”.

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➤ We prepared revised figures in which the population from Punta Laguna is indicated.

➤ We extended the discussion to emphasize the novelty of the study in order to meet the referees’ critics that explanations are not persuasive and well-structured.

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➤ We added a sentence at the end of the introduction to explicitly highlight the novelty of the study.

➤ We added some life cycle information in the revised discussion.

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➤ We changed the phrases “hypothesize” according to reviewer suggestions.

Impact Significance 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 its application as (paleo-)ecological indicator. Previous investigations addressing the impact of climate and hydrochemical regime on ostracod valve morphology have yielded 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 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~~ suggest 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.

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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 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~~2005). Their distribution is controlled by ecological factors such as salinity, temperature, and ion composition of the ambient water (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 another 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 morphometric 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.

Wrozyzna 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., [in press review 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 is new to the research of ecophenotypy in ostracods for the following reasons: ~~the investigation of the relationships between shape variations and environmental conditions are based on~~ 1\) a data-set covering a supra-regional geographical scale that coincides with the geographical range of *Cytheridella*, 2\) environmental variables include local hydrochemical data and \(regional\) climatic data, 3\) morphometric data are based on a combination of landmarks and semilandmarks that include the carapace outline and positions of pore conuli on the surface of the carapaces, and 4\) identification of environmental variables that covary with morphological changes are based on ~~two-block partial least squares analyses and multiple regression analyses~~ multivariate statistical analyses.](#)

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 Supplementary Table 1. Only adult valves were utilized in this study, providing a sufficient number of left and right valves 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 [$^{\circ}\text{C}$] (BIO1), mean diurnal range [$^{\circ}\text{C}$] (BIO2; mean of monthly maximum-minimum temperature), temperature seasonality [$^{\circ}\text{C}$] (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.

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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 Wrožyna 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.

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 3.18 (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). Using all 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 were computed based on correlation matrices.

The PLS analysis provides an idea of the overall strength of the relationships with between shape and environment. To identify the parameter(s) that affect specific morphological traits or combination of traits, multiple regression analyses were conducted on selected 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) 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 evidences 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, 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 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 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) shifts 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 indicate relationships between morphological and environmental variables, yet with different results for males and females. 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 3).

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 all analyses except male right valves; RW 3, in turn, contributes above average to variation in all cases but females right valves. 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 Methods section for consideration in the multiple regressions: RW 1 for all four datasets; RW 2 for female right and 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 (Table 1). 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 are still considered significant (see Supplementary Figure 1). Eight out of nine models are significant ($P < 0.05$); the model for RW 3 for male left valves is not ($P = 0.074$).

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: Na^+ , Ca^{2+} , Mg^{2+} , HCO_3^- , 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 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 Cl^- and SO_4^{2-} contribute significantly to many models, corresponding to differences in the hydrological regimes. Less explanation power is provided by pH, K^+ , water temperature, and mean diurnal temperature range. It is noteworthy that anions, represented by Cl^- and SO_4^{2-} , are obviously much more important than cations.

4 Discussion

Variation in temperature seasonality, annual precipitation and anions (Cl^- , 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 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 Mg^{2+} , Ca^{2+} and K^+ contents (Ramos et al., 2017) or alkalinity and sulphate, 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.

~~Although the existence of ecophenotypical characteristics is well established in ostracodology, the deduction of general relationships between shape and environmental conditions is hampered due to different approaches, geographical areas and ranges, as well as different environmental data-sets. Many studies are based on mesocosm experiments (e.g., Mezquita et al., 1999; Yin et al., 1999; van Harten, 2000; Frenzel et al., 2012). Although they have played an important role in increasing our understanding of ecophenotypical responses of ostracods to environmental changes, it is in their nature that they cannot cover the full range and interplay of natural conditions. Other authors studied field populations covering different small geographical areas with high resolution and ranges and geographical scales. Accordingly, there are either relatively small regions studied with a high resolution (e.g., van der Meeren et al., 2010); or random larger study areas/regions with widespread sampling localities disregarding that does not match the relation to the distribution of the species involved (e.g., Baltanas et al., 2002; Ramos et al., 2015; Boomer et al., 2017). Our approach is the first that covers a supra-regional scale that coincides with the geographical range of *Cytheridella* the study taxon. Another novel contribution to the investigation of ecophenotypy represents the inclusion of climatic data in contrast to many other approaches whose data-sets are often restricted to hydrochemical information. However, hydrochemical conditions usually vary on small spatial scales in contrast to climatic data. Previous studies were therefore restricted to characterize local and/or rather than regional interaction effects between of environment and on shape changes. It is known from several other organism groups that species may exhibit differences in sensitivity to ecological conditions through their geographical ranges with a higher sensitivity of range-edge populations than those nearer to the center of the species' distribution (e.g., Mills et al., 2017). Thus, some environmental parameters that were identified in other studies as major control on morphological changes (such as salinity) could be important on more restricted geographical scales due to a higher sensitivity of the local populations. The ecophenotypical response to changes of environmental conditions could vary also with geographical scale. In contrast, our approach enables to investigate the overall pattern of ecophenotypical responses to environmental changes and minimizes local effects, which may have overemphasized in studies that consider only environmental variables of the water body and/or smaller geographical scales (cf. e.g., Ramos et al., 2015).~~

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4.1 Potential environmental drivers of valve shape variation

~~This is, so far, the first approach is so far the first to study the relationship between shape and environment of in an ostracod species that occurs in tropical and subtropical areas. However, little information is available for the biology of *Cytheridella* (e.g., Meyer et al., 2017), and therefore, 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 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 ilosvayi* has a physiological origin that mirrors the varying environmental conditions.

5 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). Florida and southern Brazil are characterized by higher annual temperature gradients compared to Mexico. Annual minimum and maximum temperatures 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-
10 Data.org, 2017).

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). LifeThe time span of ostracods life cycles varies from a few months to as long as four4 years producing one or more generations per year (Horne, 2005). At least in temperature regions, the Sstart of the reproductive period (and thus the molt cycle) is in temperate regions often related to temperature (Van Doninck et al., 2003). Meyer et al. (2017) recently found that populations of *Cytheridella* from Florida may have distinctdefined calcification periods which could be that are relatedlinked to local hydrological conditions.

15 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). We expect that higher temperature seasonality induced prolonged molt cycles in populations of *C. ilosvayi* by extending intermolt periods during colder seasons. We ~~hypothesize~~ propose that 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.

25 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 poses, e.g., 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
30 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 (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 our samples 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.

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 study of 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 differences have been moreover associated with changes in Ca^{2+} , Mg^{2+} and pH (Ramos et al., 2017).

Our analyses, however, revealed correlations neither with ions related to formation of carbonate, such as HCO_3^- , Ca^{2+} , and Mg^{2+} , nor with pH. Only chloride and sulfate contents significantly correlate with carapace shape variation.

Natural sources of Cl^- in freshwater derive from marine sprays transferring NaCl into the atmosphere and are either transported as aerosol by wind or are washed out by precipitation and the weathering of rocks. Additionally, large amounts of chloride are derived anthropogenically from farming and waste water production (Müller and Gächter, 2012). 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 (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 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 Cl^- gradient extends from southeast to northwest providing generally higher chloride contents (Perry et al., 2009). Concentration gradients of SO_4^{2-} and 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 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 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 Na^+ and Cl^-) and water outwards to maintain a stable internal ionic concentration higher than that of the ambient water (Wehrauch 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 HCO_3^- outwards to maintain the internal 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

5 regulation and calcification is considered to play a key role in ostracod speciation (Mezquita et al., 1999). One of the best-studied phenomenon in ostracods is variable nodding (hollow outward flexions on the lateral surfaces on the valves) in *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 in *Cyprideis torosa* valves is pathologically and caused by 10 osmotic problems under lower salinities and lacking Ca^{2+} during molting. From the same species it is known that increasing salinity corresponds with decreasing proportion of rounded sieve pores of the valves (Frenzel et al., 2017). Considering that the variability of discrete valve traits such as nodding or sieve pore shape is related to complex physiological processes, we hypothesize-suggest that the relationship between carapace shape and ionic composition detected by our analyses could be a result of complex interplay of different physiological processes affecting valve calcification. Understanding the physiological 15 processes involved requires more detailed studies.

4.2 Genetic diversity or ecophenotypic plasticity?

Phenotypic variation in ostracods is considered to reflect either genotypic or ecophenotypic variability or a combination of 20 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; Frenzel and Boomer, 2005). The relationship between genotype and environment might differ among species, geographical 25 regions and through time (see, e.g., Sanchez-Gonzalez et al., 2004; Koenders et al., 2016). Our results clearly imply that morphological disparity 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 comprises specimens with similarly shortened valves as are found in the Floridian group. Both shortened and elongated morphotypes co-occur in one lake (i.e., Punta Laguna) (Wrozyna et al., under review). On the one hand, this co-occurrence could suggest the 30 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.

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 differences in chloride and sulfate concentrations, which are 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. A more detailed picture will require 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.

Data availability

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

Supplement link

Author contribution

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

Competing interests

The authors declare that they have no conflict of interest.

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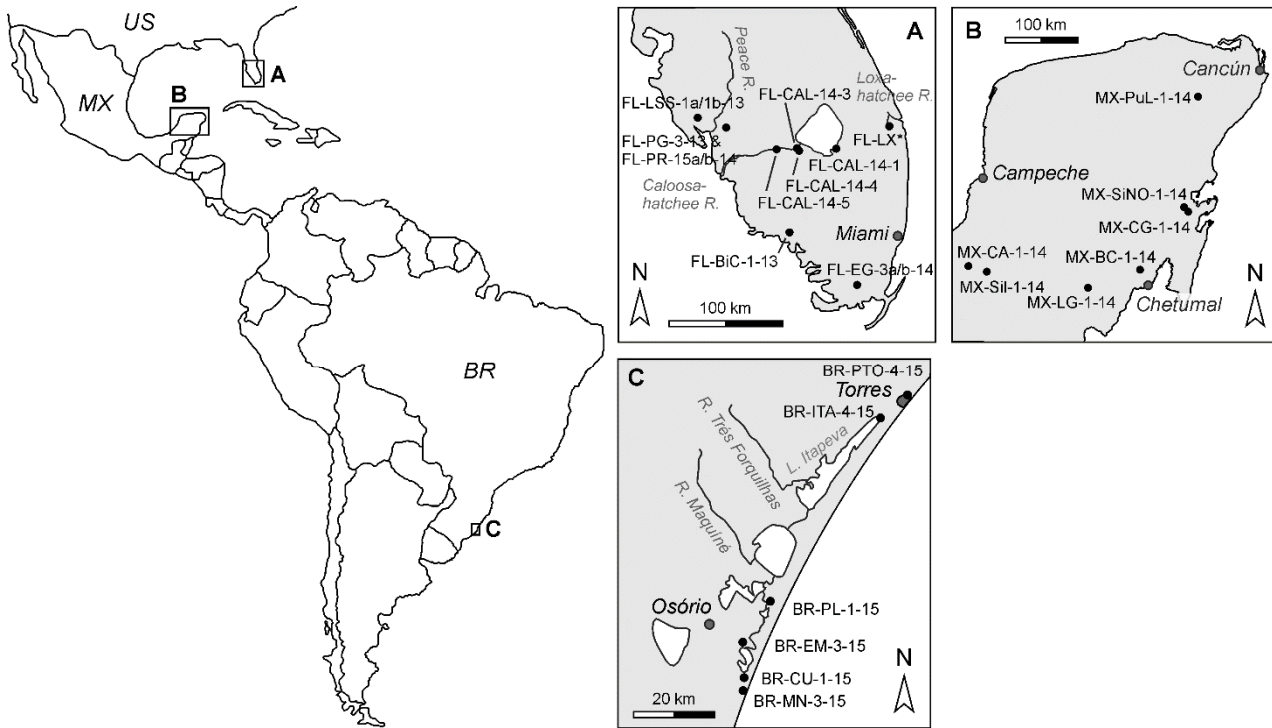
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5 **Figure 1: Geographic overview of the sampled populations (modified from Wrozyńska et al., 2016). The label FL-LX* in map A**

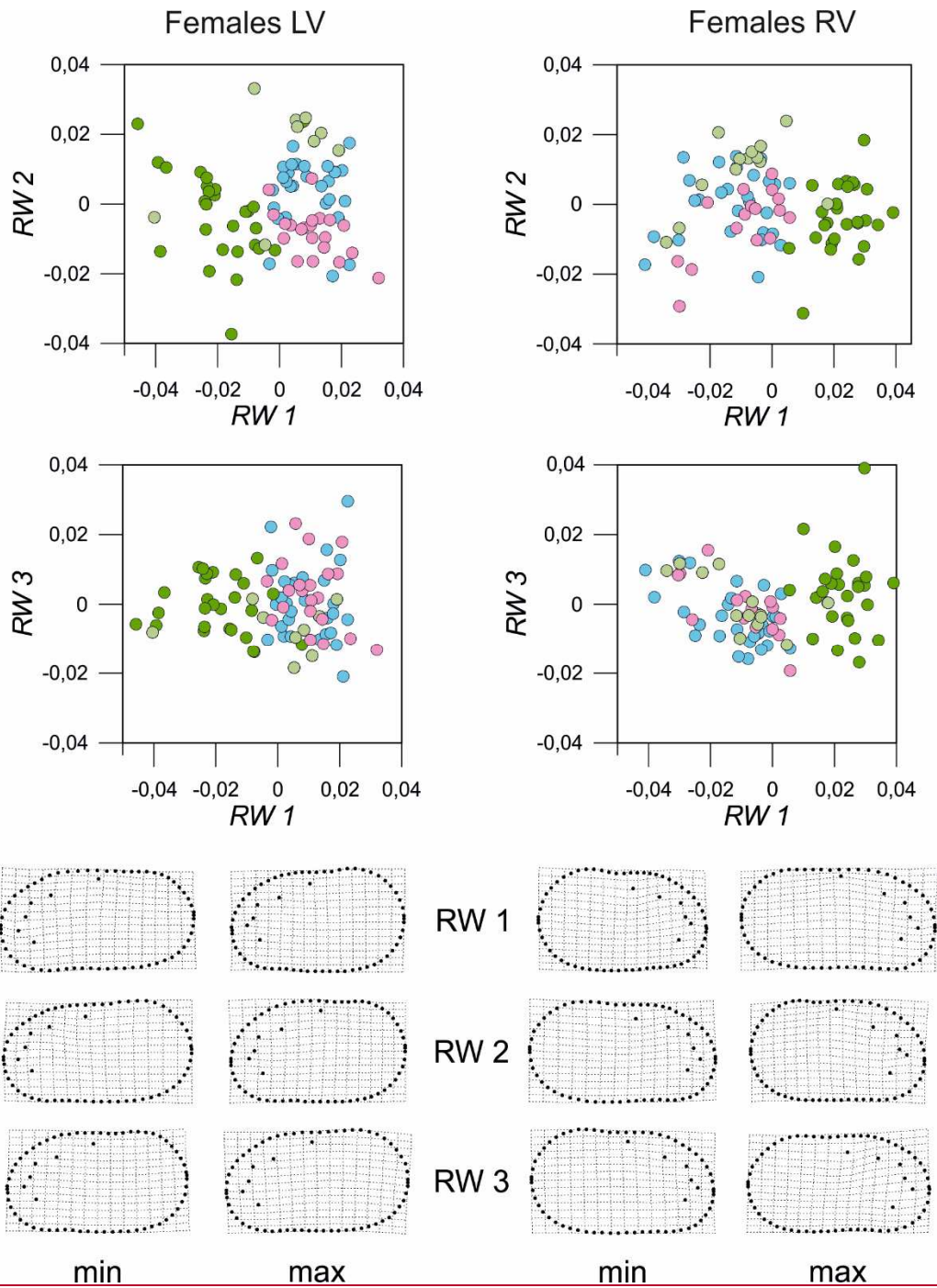


Figure 2: Relative Warps Analyses of left and right valves of females of the first three warps and the associated thin-plate splines at minimum and maximum scores. Colors refer to the different regions: blue – Florida, green – Mexico (light green – Lake Punta Laguna), pink – Brazil.

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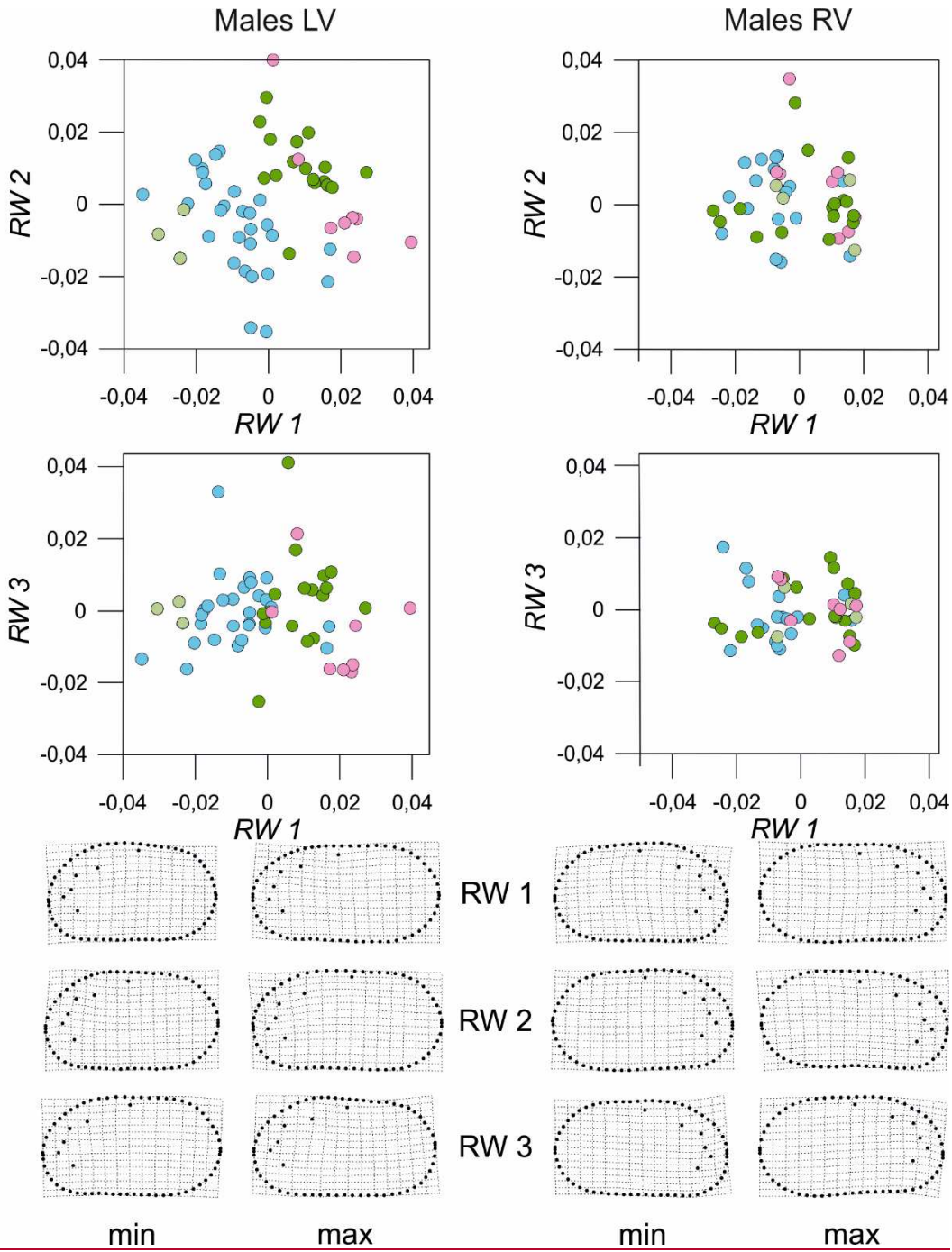


Figure 3: Relative Warps Analyses of left and right valves of males of the first three warps and the associated thin-plate splines at minimum and maximum scores. Colors refer to the different regions: blue – Florida, green – Mexico, light green – Lake Punta Laguna, pink – Brazil.

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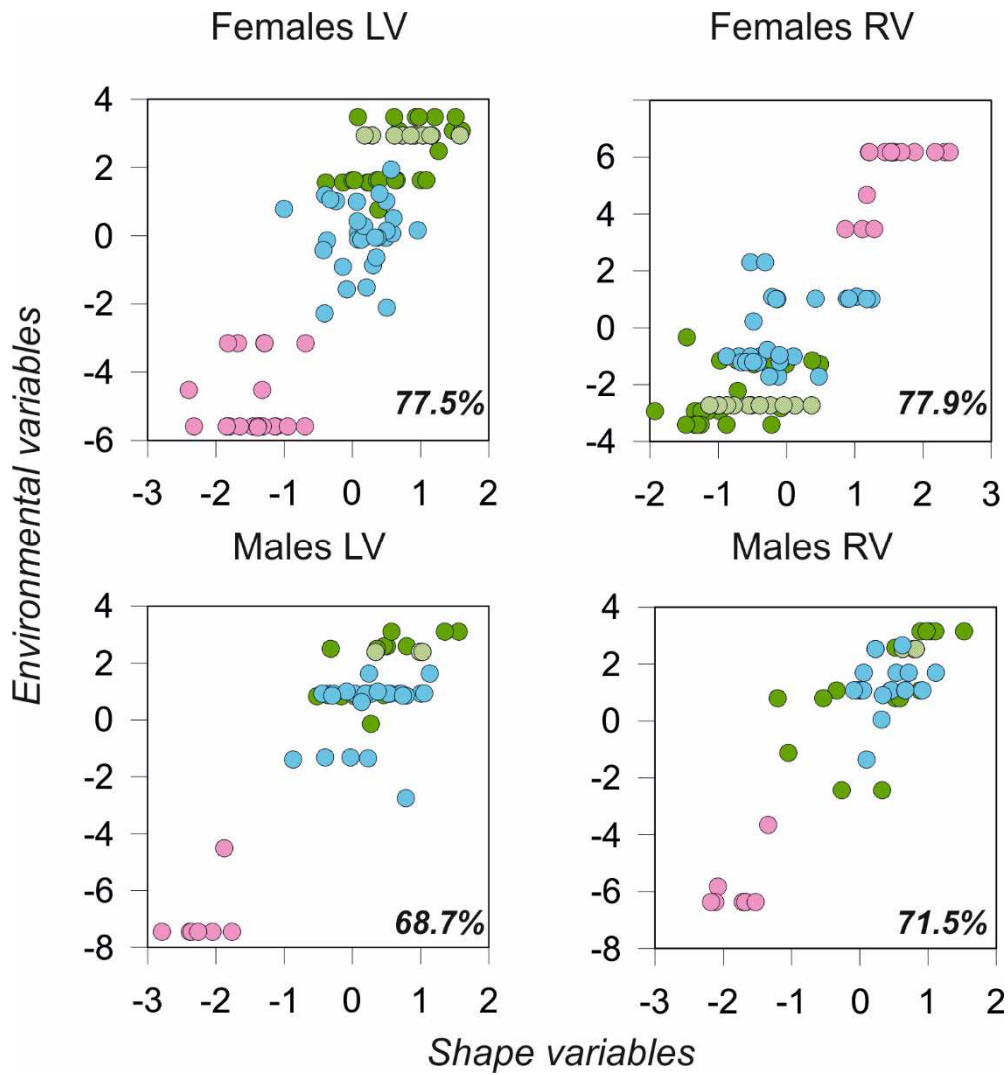


Figure 4: First axis of the PLS analysis of carapace shape and environmental variables. Colors refer to the different regions: blue – Florida, green – Mexico (light green – Lake Punta Laguna), pink – Brazil.

Table 1. Summary statistics 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 – Mean diurnal temperature range, BIO4 – Temperature seasonality, BIO12 – Annual precipitation. See Methods chapter for details.

| <i>Females LV</i> | <i>RWA</i> | | <i>PLS</i> | <i>Multiple Regressions</i> | | | |
|-------------------|-------------------------|--|---------------------------------|------------------------------------|----------|-------------------------------|---|
| | % var. explained | Reification | Loadings axis 1 (77.55%) | R²_{adj} | P | Shapiro-Wilks test (P) | Predictor variables |
| RW 1 | 33.22 | elongate-elliptical vs. short asymmetrical with distinctly inflated posterior region; position of pore conuli | -0.5637 | 0.632 | <0.001 | 0.0200 | BIO4 (46.2%), SO ₄ (19.3%), pH (13.0%), Cl ⁻ , Temp., BIO12 |
| RW 2 | 19.00 | elongate-elliptical with slightly inflated posterior region vs. short; position of pore conuli | 0.4798 | 0.510 | <0.001 | 0.0063 | BIO12 (43.9%), Cl ⁻ (32.9%), K (11.7%), SO ₄ , BIO4 |
| RW 3 | 10.72 | minor deviations of outline; small differences in position of pore conuli | -0.1850 | 0.142 | 0.002 | 0.3338 | SO ₄ (33.3%), Temp. (27.5%), Cl ⁻ (24.6%), BIO4 (14.6%) |
| | | | | | | | |
| <i>Females RV</i> | <i>RWA</i> | | <i>PLS</i> | <i>Multiple Regressions</i> | | | |
| | % var. explained | Reification | Loadings axis 1 (77.95%) | R²_{adj} | P | Shapiro-Wilks test (P) | Predictor variables |
| RW 1 | 40.39 | elongate-elliptical vs. short asymmetrical with distinctly inflated posterior region; position of dorsal dip point of the posterior curvature; position of pore conuli | -0.3892 | 0.593 | <0.001 | 0.0079 | BIO4 (44.5%), Cl ⁻ (20.4%), SO ₄ (18.1%), Temp., K, BIO12 |
| RW 2 | 14.89 | elongate with slightly inflated posterior region vs. short; position of dorsal dip point of the posterior curvature; position of pore conuli | -0.2373 | 0.257 | <0.001 | 0.0040 | BIO12 (48.4%), SO ₄ (23.5%), Cl ⁻ (20.1%), BIO4 |
| RW 3 | 10.50 | elongate-elliptical vs. short asymmetrical with inflated posterior region; position of dorsal dip point of | 0.1003 | <i>n/a</i> | | | |

| | | | | | | | |
|-----------------|-------------------------|--|---------------------------------|------------------------------------|----------|-------------------------------|---|
| | | the posterior curvature; position of pore conuli | | | | | |
| Males LV | RWA | | PLS | Multiple Regressions | | | |
| | % var. explained | Reification | Loadings axis 1 (67.74%) | R²_{adj} | P | Shapiro-Wilks test (P) | Predictor variables |
| RW 1 | 24.14 | elongate-elliptical vs. short asymmetrical with slightly inflated anterior part; position of pore conuli | -0.4063 | 0.638 | <0.001 | 0.3948 | BIO4 (37.3%), Cl ⁻ (34.5%), BIO12 (15.0%), SO ₄ (10.8%), pH |
| RW 2 | 22.25 | elongate vs. short with higher dorsal margin; position of pore conuli | 0.2359 | n/a | | | |
| RW 3 | 13.92 | minor deviations of outline; position of pore conuli | 0.2792 | 0.042 | 0.074 | 0.0017 | (not significant) |
| Males RV | RWA | | PLS | Multiple Regressions | | | |
| | % var. explained | Reification | Loadings axis 1 (71.55%) | R²_{adj} | P | Shapiro-Wilks test (P) | Predictor variables |
| RW 1 | 29.88 | elongate-elliptical vs. short-asymmetrical with slightly inflated anterior part; position of pore conuli | -0.3798 | 0.318 | 0.001 | 0.2221 | BIO12 (31.8%), BIO4 (28.7%), Cl ⁻ (15.8%), pH (12.5%), K (11.2%) |
| RW 2 | 12.81 | elongate vs. short with higher dorsal margin; position of dorsal dip point of the posterior curvature; position of pore conuli | 0.0157 | n/a | | | |
| RW 3 | 11.98 | shape of anterior part; position of pore conuli | 0.5502 | 0.286 | <0.001 | 0.2978 | BIO4 (57.2%), BIO2 (42.8%) |