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# Original Article

## Adaptive shell-morphological differences and differential fitness in two morphospecies of *Lanistes* (Gastropoda: Ampullariidae) from the northern region of the Malawi Basin

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### ABSTRACT

Environment–phenotype correlations are a central pattern of adaptive radiations, but such correlations and the expected associated reciprocal differences in fitness of divergent phenotypes in their respective niches are rarely demonstrated. Ecophenotypic plasticity may result in environment–phenotype correlations too, however, so that certain observed morphological differences, even in adaptive radiations, may result from plasticity instead of differential adaptation. Here, we examine the nature of observed morphological differences in two morphospecies of *Lanistes* gastropods from the northern region of the Malawi Basin that are part of an ongoing adaptive radiation. We subjected experimental populations of each of these two morphospecies, i.e. *L. sp. (solidus-like)*, which occupies intralacustrine habitats with comparatively stable environmental conditions, and *L. sp. (ovum-like)*, which occurs in satellite waterbodies with fluctuating environmental conditions, to a common garden experiment in the laboratory. Our experimental conditions approximated those of stable, intralacustrine habitats. We monitored fecundity and subjected wild-caught parents and intraspecific  $F_1$  offspring to semilandmark geometric morphometrics. *L. sp. (solidus-like)* exhibited higher fecundity than *L. sp. (ovum-like)*. Whereas wild-caught parents of both morphospecies had similar shell sizes, 9-month-old  $F_1$  offspring of *L. sp. (solidus-like)* were significantly smaller than those of *L. sp. (ovum-like)*, indicating slower growth. Substantial shell-morphological differences were observed between the two morphospecies in the wild-caught parents as well as in  $F_1$  offspring, indicating that shell-morphological differences are genetically inherited. Our results also indicate differential fitness but fully reciprocal transplant experiments would be required to demonstrate local adaptation.

**Keywords:** ongoing adaptive radiation; geometric morphometrics; common garden experiment; *Lanistes*; East African Rift System; Lake Malawi; fitness

### INTRODUCTION

Adaptive radiations represent the rapid evolution of various new species from a common ancestor as a consequence of adaptation to distinct ecological niches (Schluter 2001a, Salzburger *et al.* 2014). Various species evolving in such radiations differ in key phenotypic traits that correlate with major aspects of environmental variation, so that species-specific trait combinations facilitate the exploitation of a certain ecological niche (Schluter 2001a, De-Kayne *et al.* 2024). The proposed causal relationship

between diversification and ecological differentiation implies that local adaptation occurred repetitively across populations (Kawecki and Ebert 2004) and contributed to lineage divergence, as opposed to scenarios where ecological differentiation postdates diversification or evolves as a by-product of speciation. If various local populations or demes simultaneously adapt to distinct niches, each deme will evolve phenotypic traits that enhance performance (or trait ‘utility’) in its respective niche compared to in other niches, so that the demes reciprocally

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display higher fitness in their niche/habitat compared to demes originating from other niches/habitats. Under such conditions, divergent natural selection driving the divergence in phenotypic traits may simultaneously drive reproductive isolation, either prezygotically, by reducing/restricting mating opportunities among niches/habitats, or postzygotically (Schluter 2001b). Postzygotic isolation may be intrinsic, i.e. due to genetic or sexual incompatibilities (Coyne and Orr 2004), and/or extrinsic, depending on the interaction of genotypes in their environments (Schluter 2001b, Nosil 2012). Rare matings among differently adapted, isolated demes could result in hybrids with intermediate phenotypes, exhibiting reduced fitness in each of the available ecological niches, which may reinforce isolation.

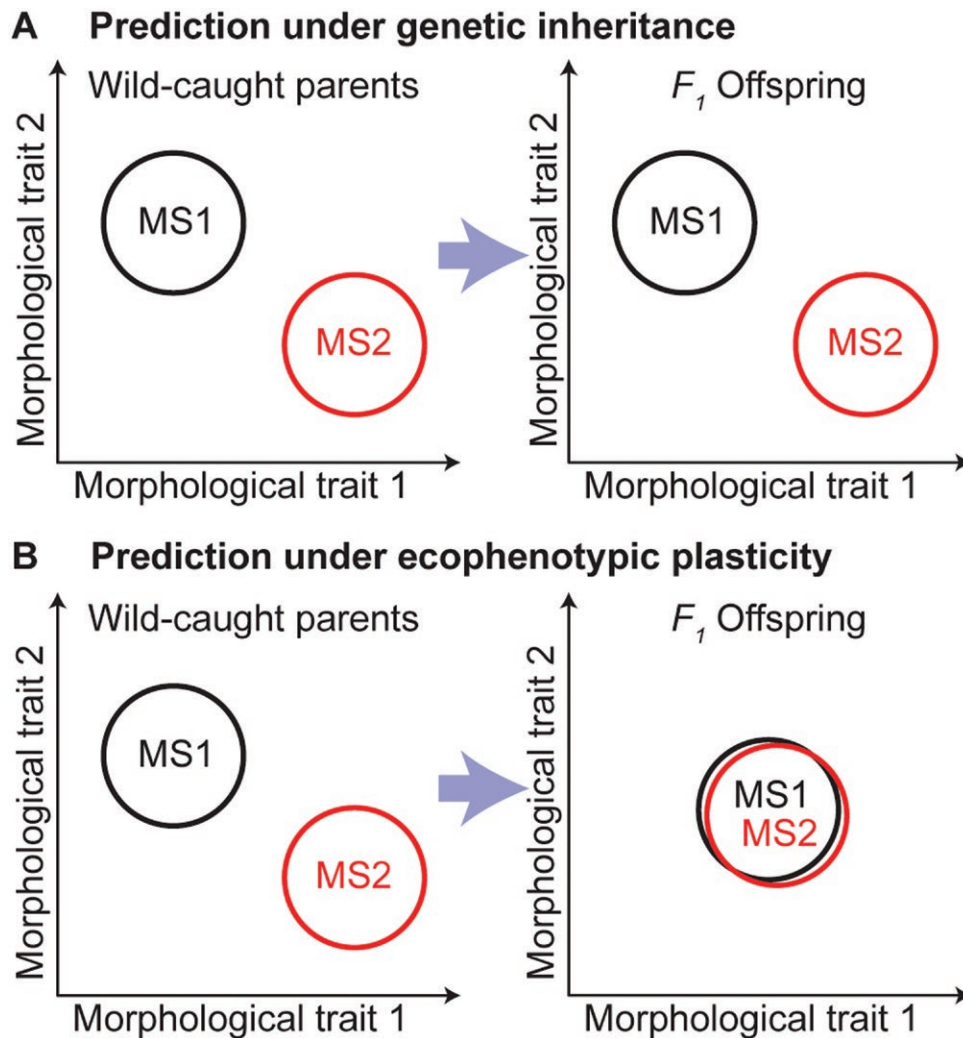
Within this theoretical framework, testing whether a species flock has evolved via adaptive radiation requires verification of various key hypotheses. A species flock is defined as a group containing various species that are endemic to a particular, small area, that are ecologically diverse and that originated from a single ancestor species. These hypotheses include that the species evolved (i) at a rate that is (typically) elevated compared to diversification rates in related clades that did not evolve via adaptive radiation, (ii) with descendant species displaying phenotype–environment correlations that have some genetic basis, resulting in (iii) reciprocally enhanced performance and thus fitness in their local niche/habitat compared to closely related species from other niches and habitats. Furthermore, a substantial number of the descendant species should have evolved via ecological speciation, implying that phenotypic divergence is directly linked to the establishment of reproductive barriers. Whereas most or all of these requirements have been examined or tested for hallmark adaptive radiations, such as cichlid fishes (Wagner *et al.* 2012, Ronco *et al.* 2021), Darwin's finches (Grant and Grant 2008), and *Anolis* lizards (Losos 2009), environment–phenotype correlations have been suggested in various other radiations, but they are rarely demonstrated using quantitative environmental and phenotypic data (De-Kayne *et al.* 2024). Moreover, fitness differentials of divergent phenotypes in their respective niches are rarely demonstrated, as they often require challenging experiments or direct measurements of the effects of selection in nature (Schluter 2001a, Kawecki and Ebert 2004).

Various candidate systems of adaptive radiation exist in freshwater invertebrates, notably from ancient lake systems, where several taxa have radiated in parallel (Sherbakov 1999, West and Michel 2000, West *et al.* 2003, von Rintelen *et al.* 2004, 2010), but demonstrating that adaptation to distinct niches fuelled diversification is particularly challenging in these candidates. One such candidate system is the ongoing evolutionary radiation of *Lanistes* gastropods from the Malawi Basin (Mandahl-Barth 1972, Berthold 1990a, 1990b, Schultheiß *et al.* 2009), representing an endemic clade with five nominal species that evolved over the last ~100 kya (Van Bocxlaer 2017), and thus since the last megadrought that caused massive lake level drops of Lake Malawi (Cohen *et al.* 2007). Diversification rates in this clade are elevated, as *Lanistes* is estimated to have evolved since ~50 Mya (Mahulu *et al.* 2021) and contains 43 valid species (Cowie 2015). Studies of genetic and morphological variability in 15 populations comprising specimens of four of the five nominal species of *Lanistes* from the Malawi Basin indicated that the

strongest genetic differences occur between populations from the northern and southern regions (Van Bocxlaer 2017). This divergence is due to a single geographical barrier, namely steep rocky shorelines along the centre of the Malawi Rift, which are uninhabitable for *Lanistes* and, therewith, prevent migration between regions. Within each region two distinct gene pools coexist and phenotypic divergence along a depth gradient was found to be positively correlated with neutral genetic differentiation (Van Bocxlaer 2017), generating a pattern of 'isolation-by-adaptation' (see Nosil *et al.* 2008, Orsini *et al.* 2013). Regarding shell morphology, the four gene pools do not coincide well with the four nominal species and these gene pools display varying degrees of overlap in morphospace, with the main morphological differentiation occurring between stable, intralacustrine, and fluctuating, satellite habitats (Van Bocxlaer 2017). A common garden experiment with three of the nominal species of this *Lanistes* radiation from the southern region of the Malawi Basin indicated significant differences in survival and fecundity among these species in contrasting habitats, and most of the morphological differences among these species persisted in two offspring generations in a controlled environment, demonstrating that the divergence in shell morphology is genetically inherited and associated with marked fitness differences among species (Van Bocxlaer *et al.* 2020).

Thus far, *Lanistes* from the northern region of the Malawi Basin have been studied less extensively than those from the southern region (e.g. Louda *et al.* 1982, 1984, Berthold 1990a, 1990b), but observed patterns differ substantially from those in the southern region. Three morphospecies occur in the northern region, namely *Lanistes* sp. (*solidus*-like), *L.* sp. (*ovum*-like), and *L.* sp. (*ellipticus*-like), although as mentioned, they are genetically differentiated from identically named morphospecies from the southern region, implying that taxonomic revision is required. Within the northern region these three morphospecies are genetically assigned to two gene pools that have always been retrieved in direct sympatry and that overlap completely in shell morphology (Van Bocxlaer 2017). Nevertheless, assignments of individuals from the northern region to one of both gene pools consistently receive high assignment probabilities, indicating that this genetic structure is strong and robust. The observed pattern in the northern region contrasts with that in the southern region, where gene pools are rarely found sympatrically and display limited morphospace overlap.

Here we study two of these three morphospecies from the northern region, namely *L.* sp. (*solidus*-like), which occurs in comparatively stable, intralacustrine habitats, and *L.* sp. (*ovum*-like), which occurs in satellite waterbodies that display fluctuating environmental conditions. We study shell morphology of these morphospecies in a common garden experiment to examine whether morphological differences persist in  $F_1$  raised in controlled conditions. If differences persist, then it would imply that phenotypic differences are largely genetically inherited, whereas they can be attributed to ecophenotypic plasticity if differences disappear upon raising offspring in controlled environments (Fig. 1). Our experiment thus allows us to characterize the nature of environment–phenotype correlations in two morphospecies from an ongoing adaptive radiation of *Lanistes* gastropods.



**Figure 1.** Graphical representation of our theoretical predictions. A, if morphological differences among two morphospecies, here labelled as MS1 and MS2, are genetically inherited, then we expect that these differences will persist in offspring generations raised in controlled laboratory environments. B, alternatively, morphological differences between the two morphospecies may be due to ecophenotypic plasticity, in which case we expect these differences to disappear if offspring are raised under controlled laboratory conditions that effectively eliminate the environmental differences between habitats.

## MATERIAL AND METHODS

### Sampling and specimen handling

*Lanistes* sp. (*ovum*-like) was collected from pools at the Mutakisi River (which temporarily connects to the Songwe River during the rainy season) and a backwater pool of the South Rukuru River, both within a distance of 3 km of the shoreline of Lake Malawi. *Lanistes* sp. (*solidus*-like) was collected at a depth of ~0.5 m from within Lake Malawi near Ngara. Specimens were kept in population-specific containers/bags during transfer to the lab, where they were first placed in pre-installed aquaria (one aquarium per sampling locality) for an acclimatization period of ~6 months to diminish potential maternal effects, even though such effects appear to be very small in *Lanistes* from the Malawi Basin (Van Bocxlaer *et al.* 2020). After the acclimatization period, we constructed experimental populations using individuals from the various sampling localities, creating two replicates for each morphospecies. We initially had 10 and 14 wild-caught specimens of *L.* sp. (*solidus*-like) and *L.* sp. (*ovum*-like), respectively, per experimental population.

*Lanistes* is dioecious, with internal fertilization, and females have a seminal receptacle (Berthold 1991), in which they may store sperm from previous matings. As we collected adult individuals, we needed to ensure that previous matings in the wild do not contribute to offspring in our experiment, particularly if nonassortative matings among morphospecies would occur in the wild, which could homogenize morphology. We avoided effects of nonassortative mating by selecting sampling localities where only one morphospecies occurred as source for our experiment. How long female *Lanistes* can preserve sperm in their seminal receptacle is poorly known, but females of *Pomacea* can produce fertilized eggs for several weeks after their last copulation (Albrecht *et al.* 1999). Although we have never observed similar capabilities for *Lanistes* (Van Bocxlaer *et al.* 2020), we avoided effects of delayed fertilization by a long acclimatization phase. Finally, sperm competition occurs in ampullariids and mate-switching experiments have demonstrated a swift turnover of days and up to a few weeks in the paternity of offspring towards the most recent partner (Yusa 2004). Therefore, our final



precaution to avoid an influence of pre-experimental matings was to remove egg clusters that were formed in the first month after initialization of our experiment.

### Experimental conditions

Each experimental population was kept in an aquarium of 60 L with 3–4 cm of sterilized river sand, some small stones, a piece of hardwood, a jar with coral sand to buffer water hardness, and some submerged aquatic macrophytes, which may serve both as substrate to deposit eggs and as food. As macrophytes were quickly eaten, they were replaced with plastic macrophytes that serve solely as substrate for egg deposition. Aquaria were filled with a mix of 50% tap water and 50% osmotic water that was further treated with pH-plus (JBL, Neuhausen, Germany) and AquaSafe (Tetra). Each tank had a 75-W heating element, a Biopower 160 filter, and a single air diffuser with a flow of 100 L/h (Eheim, Deizisau, Germany). Each aquarium further contained a few plecostomid fishes (*Ancistrus gastrosteus*) from a commercially obtained inbred lineage to help maintain the ecological equilibrium in aquaria. Animals were fed daily with JBL Novo Pleco chips, occasionally supplemented with vegetables (carrot, cucumber, lettuce, potato). Pumps were cleaned as needed to guarantee a continuous high level of mechanical and biological filtration and water changes took place every 2–3 weeks to avoid the buildup of metabolites. Water conditions were pH = 7.40 ± 0.36, EC (electrical conductivity) = 721 ± 215 µS/cm, and temperature = 26.96 ± 0.91°C, which reflect mean diurnal natural conditions in our sampling localities well, except that conductivity was approximately doubled to prevent shell corrosion. Fluctuations in these conditions are limited compared to fluctuations in satellite waterbodies in Malawi, and therefore reflect the stable, intralacustrine habitat better than they do fluctuating satellite waterbodies. We used a photoperiod of 8 h/day.

An unanticipated complication of our experiment was that *L. sp.* (*ovum*-like), the morphospecies that occurs in satellite waterbodies with fluctuating environmental conditions, did not reproduce spontaneously in contrast to *L. sp.* (*solidus*-like). Therefore, we examined during the acclimatization phase various environmental cues that may trigger reproduction. This exploration indicated that exposure to an artificial heat wave of 30–31°C for several consecutive days (during which fishes were removed from the aquarium) induced reproduction in *L. sp.* (*ovum*-like). During the experiment both morphospecies were subjected to such heat waves to maintain similarity in environmental conditions among morphospecies and replicates.

### Geometric morphometrics

We imaged specimens in apertural view with a Canon Eos 700D D-SLR camera equipped with a 60-mm EFS macro lens. Parents were imaged just before the experiment,  $F_1$  offspring after having been raised for ~9 months. Each specimen image contained a scale bar, allowing to calibrate the size of pixels. Such images were compiled in a .tps file with TpsUtil v.1.75 (Rohlf 2018), for digitization of 11 landmarks and four open semilandmark curves per specimen in TpsDig v.2.31 (Rohlf 2017). These open semilandmark curves were anchored between two landmarks each and they were resampled by length to obtain 20, 40, 20, and 15 equidistant semilandmarks, respectively. After further

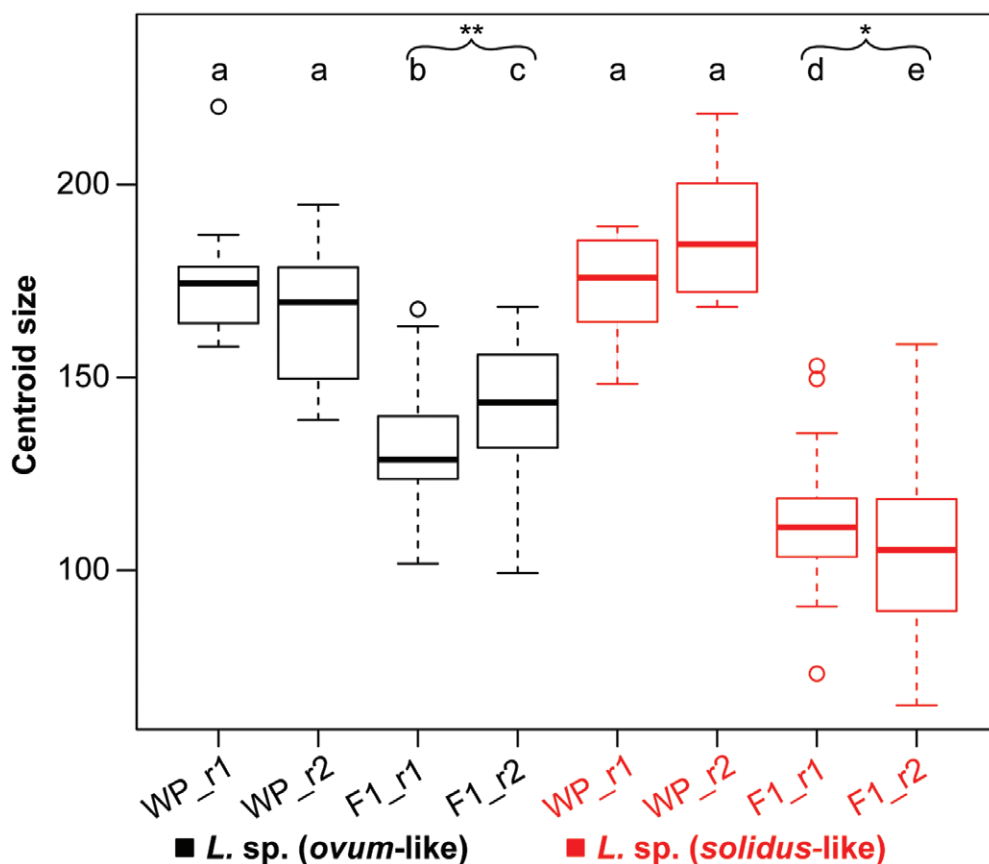
conversion with TpsUtil, we imported the digitized coordinates into CoordGen8 of the Integrated Morphometrics Package (Sheets 2014). As images were size-calibrated, the centroid size, i.e. a geometric measurement of size variation, unambiguously reflected specimen size. Variation in position, orientation and scale was removed via Procrustes superimposition after which we identified helper points to slide semilandmarks along their curves in SemiLand8 using perpendicular projection to the reference of the entire dataset. Subsequently, our dataset of centroid size and partial Procrustes superimposition coordinates (Van Bocxlaer et al. 2024) was imported into R v. 4.1.2. (R Core Team 2021) for further statistical analyses.

### Fecundity and statistical analyses

Fecundity in our experiments was documented as the number of juveniles produced per population that survived ~9 months after emergence from the eggs. We analysed these count data with a  $\chi^2$  test. Shell size was analysed as centroid size among morphospecies, generations, populations, and replicates. Normality of size data was verified with Shapiro–Wilk tests and the homogeneity of variances with a Bartlett test. Because some of the assumptions of parametric tests were violated, we performed nonparametric Kruskal–Wallis tests and post-hoc pairwise Wilcoxon rank sum tests with Bonferroni correction to compare size differences.

The geometric morphometric data (excluding centroid size) were subjected to nonmetric multidimensional scaling (nmMDS) with 1000 random starting configurations using functions of the packages MASS v.7.3-54 (Venables and Ripley 2002) and VEGAN v.2.5-7 (Oksanen et al. 2020). Stress values, which indicate the goodness-of-fit of these ordinations, were multiplied by 100 and evaluated using the criteria of Kruskal (1964) and Clarke (1993). Stress values ≤ 10 indicate that ordination on a few axes is highly representative for the higher-dimensional data, with limited risk of making false inferences as higher axes are discarded, whereas higher stress values imply that such risks are increasing. Additionally, we examined whether any systematic changes occur in the morphospace occupation upon using nmMDS in two or three dimensions. Upon ordination, we examined shape changes in the morphospace occupation space with functions of GEOMORPH v.4.0.1 (Adams et al. 2021, Baken et al. 2021).

Patterns in morphospace occupation were analysed with multivariate statistical testing. We evaluated the assumptions of parametric tests, namely multivariate normality and the homogeneity of the variance–covariance for each group. We tested multivariate normality with an E-test using 1000 bootstrap replicates in ENERGY v.1.7-9 (Rizzo and Szekeley 2017). Multivariate homogeneity of group variances was evaluated with the *betadisper* function of VEGAN, and the pairwise equality of variance–covariance matrices with our own implementation of Box’s M test. As some assumptions for parametric testing were not met, we eventually used nonparametric permutation tests to examine whether the centroids (multivariate means) and dispersion of populations were equal. We performed a permutational multivariate analysis of variance (perMANOVA) with 10 000 permutations on distance matrices using the *adonis* function of VEGAN and we confirmed the robustness of these results by comparing the test statistics of 10 000 MANOVA tests on permuted



**Figure 2.** Boxplots of centroid size among wild-caught parents (WP) and lab-reared  $F_1$  offspring ( $F_1$ ) of *Lanistes* sp. (*ovum*-like) and *L. sp.* (*solidus*-like). Replicates are indicated as r1 and r2, respectively. Significance levels are indicated with letters at the top: different letters indicate highly significant differences ( $P < .001$ ), except for when indicated with \*\* ( $.001 < P < .01$ ) and \* ( $.01 < P < .05$ ).

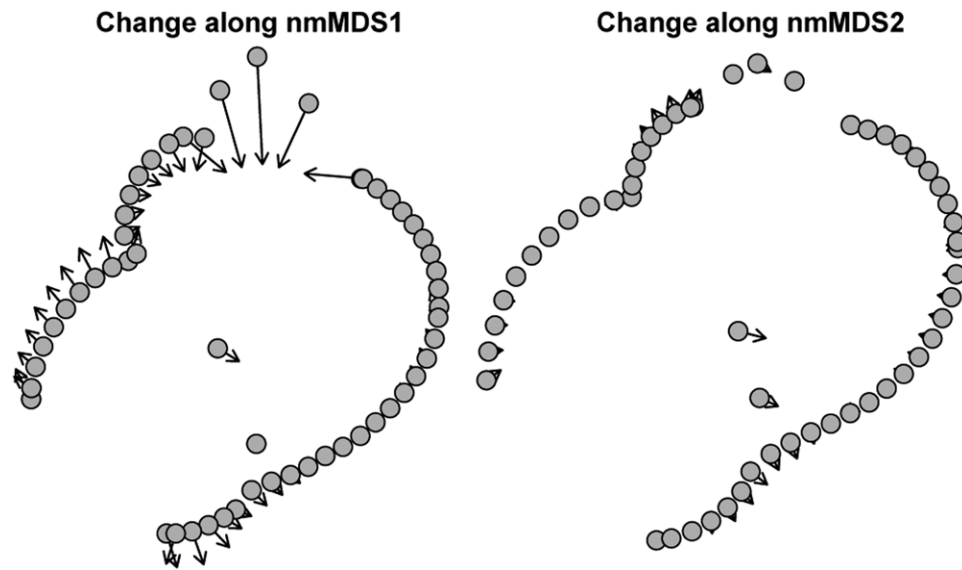
datasets to that of the actual dataset. Post-hoc pairwise comparisons were organized with permutational Hotelling  $T^2$  tests with 10 000 permutations and Bonferroni correction in HOTELING v.1.0-8 (Curran 2017). Furthermore, we examined patterns of morphospace occupation with model-based clustering using Gaussian finite mixture models in MCLUST v.5.4.9 (Fraley and Raftery 2007, Scrucca et al. 2016), which allows identifying groups based on mathematical descriptors of the variance-covariance structure of the data in the absence of a priori group assignments. Modelling was performed with the expectation-maximization algorithm and a Bayesian Information Criterion (BIC) was used to evaluate model support. We performed these analyses with 14 models that parameterize the multivariate within-group covariance. These models differ in the distribution of the covariance (spherical, diagonal, ellipsoid) as well as the volume (equal or variable), the shape (equal or variable), and the orientation (none, equal, variable, or along coordinate axes) of within-group variance.

## RESULTS

Due to some mortality, our experimental populations of wild-caught parents contained 11 and 14 versus 9 and 8 individuals at the onset of reproduction for both replicates of *L. sp.* (*ovum*-like) and *L. sp.* (*solidus*-like), respectively. There was some delay in reproduction in certain aquaria, but we obtained 177

offspring individuals in total that survived for at least 9 months: for *L. sp.* (*ovum*-like) replicates 1 and 2 we obtained 48 and 26  $F_1$  offspring individuals, respectively, compared to 49 and 54  $F_1$  offspring individuals in replicates 1 and 2 of *L. sp.* (*solidus*-like), respectively (Van Bocxlaer et al. 2024). *L. sp.* (*solidus*-like) displayed significantly greater fecundity than *L. sp.* (*ovum*-like) ( $\chi^2$  test;  $\chi^2 = 4.524$ , d.f. = 1,  $P = .033$ ). The per-capita fecundity of *L. sp.* (*ovum*-like) was substantially lower than that of *L. sp.* (*solidus*-like) (mean  $\pm$  SD;  $3.11 \pm 1.77$  vs.  $6.10 \pm 0.92$ ).

Examining centroid size, a measure of shell size, we could not reject normality, except for *L. sp.* (*ovum*-like) parent pop. 1 (Shapiro-Wilk test;  $W = 0.823$ ,  $P = .019$ ). We found significant differences in centroid size among generations, and among populations, but not among taxa (Fig. 2; Kruskal-Wallis tests; taxa:  $\chi^2 = 55.891$ , d.f. = 1,  $P < .001$ ; generation:  $\chi^2 = 92.104$ , d.f. = 1,  $P < .001$ ; populations:  $\chi^2 = 60.531$ , d.f. = 3,  $P < .001$ ; replicates:  $\chi^2 = 0.319$ , d.f. = 1,  $P = .573$ ). Upon performing pairwise comparisons, we did not find any significant differences in shell size in the parent populations, but shell size differences were highly significant among  $F_1$  offspring of *Lanistes* sp. (*ovum*-like) and *L. sp.* (*solidus*-like) ( $\chi^2 = 73.007$ , d.f. = 1,  $P < .001$ ). At 9 months of age, these  $F_1$  offspring remained significantly smaller than the wild-caught parents: those of *L. sp.* (*ovum*-like) reached on average  $\sim 78\%$  of the parent size, whereas those of *L. sp.* (*solidus*-like) reached only  $\sim 62\%$ . Additional significant differences in shell size were observed between replicates of *L. sp.*



**Figure 3.** Shape changes along nmMDS1 and 2. The configuration of circles indicates the shape at the negative extreme of the axis with arrows indicating displacements towards the positive extreme of the same axis, while maintaining a position at the origin on the other axis (shape changes along nmMDS1 are illustrated at nmMDS2 = 0 and vice versa).

(*ovum*-like) and *L. sp. (solidus*-like), but they were much smaller than the differences among parents and offspring as well as those among  $F_1$  offspring of both morphospecies (Fig. 2). As the sample sizes of wild-caught parents versus  $F_1$  offspring differed, substantial differences existed in statistical power depending on generations. Therefore, we re-performed these tests on  $F_1$  offspring after resampling to the same sample sizes as their wild-caught parents and bootstrapped this procedure to establish distributions of the resulting statistics. This procedure indicated that differences between  $F_1$  offspring replicates of *L. sp. (solidus*-like) disappeared upon resampling (pairwise Wilcoxon tests; mean  $\pm$  SD:  $W = 44.13 \pm 9.04$ ;  $P = .475 \pm .306$ ), which was often but not always the case for *L. sp. (ovum*-like) ( $W = 43.96 \pm 14.58$ ;  $P = .158 \pm .218$ ). Differences between  $F_1$  offspring of both morphospecies consistently remained significant upon such resampling, however ( $W = 379.46 \pm 17.92$ ;  $P < .001$ ).

### Geometric morphometrics

Upon performing nmMDS in two dimensions, we observed limited stress (7.529), which was only slightly reduced for nmMDS in three dimensions (5.052), indicating a strong goodness-of-fit. Comparisons of the morphospace occupation of both of these analyses indicated that differences were small, but that part of the variation was compacted along the second axis in the 3D analysis (not shown), indicating that the interpretation of nmMDS in 2D is robust with limited chances for misinterpretation. Examination of the contribution of variables to the overall morphospace occupation indicated that almost all variables had a significant effect. Morphological differences in shell shape along nmMDS1 were much more marked than along nmMDS2, and reflected mainly differences in the height of the apex, the size of the aperture compared to the total size of the shell, and how pronounced shoulders are. Along nmMDS2 changes mainly reflected the position of the umbilicus (Fig. 3).

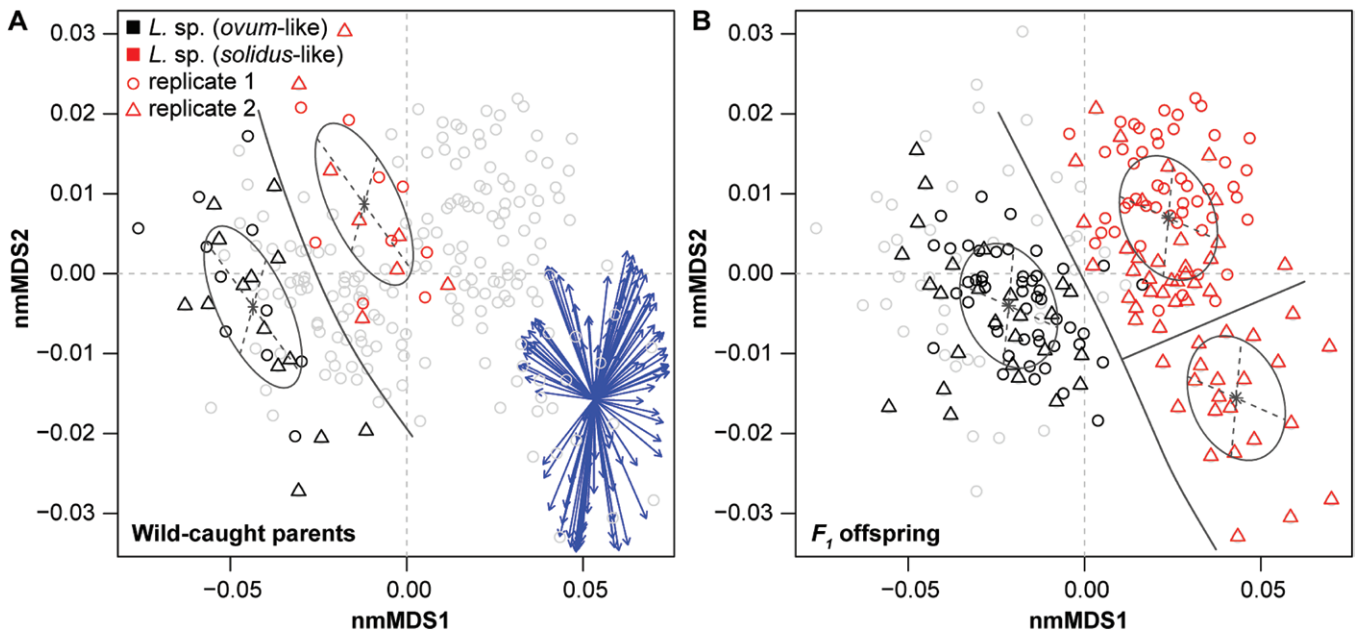
The morphospace occupation of wild-caught parents indicated strong morphological differentiation between *L. sp.*

(*ovum*-like) and *L. sp. (solidus*-like), without morphospace overlap (Fig. 4A). These differences persisted in the  $F_1$  offspring, but slight morphospace overlap was observed in this generation. The existence of such overlap in the  $F_1$  generation versus none in the wild-caught parent generation probably results from the inclusion of more specimens in the  $F_1$  compared to parent generation, because the total morphospace distance between the geometric means of populations of *L. sp. (ovum*-like) and *L. sp. (solidus*-like) was statistically significantly smaller in the wild-caught parents than in the  $F_1$  offspring (mean  $\pm$  SD:  $0.03588 \pm 0.00309$  vs.  $0.05134 \pm 0.00577$ , respectively; Kruskal–Wallis test;  $\chi^2 = 5.333$ , d.f. = 1,  $P = .021$ ). For both morphospecies, the  $F_1$  offspring individuals were displaced compared to the morphospace occupation of wild-caught parents, but these displacements were proportionally larger for *L. sp. (solidus*-like) (Fig. 4). There were slight differences in the morphospace occupation of  $F_1$  offspring between the replicates of *L. sp. (ovum*-like), but more so for *L. sp. (solidus*-like) (Fig. 5).

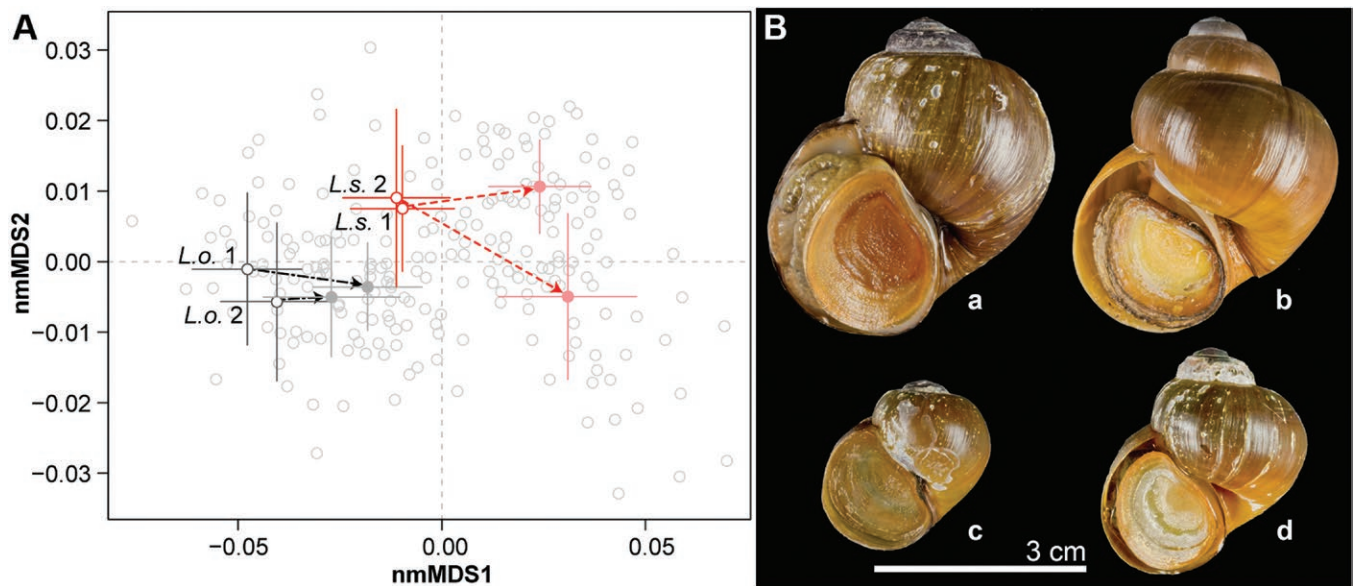
Multivariate normality was not rejected for any of the populations per morphospecies, but upon pooling replicates, normality was rejected for  $F_1$  offspring of *L. sp. (solidus*-like) ( $e = 1.004$ ,  $P = .031$ ). The homogeneity of the variances was not rejected. In-depth statistical comparisons revealed highly significant differences among generations and among morphospecies (perMANOVA; generations:  $F = 54.353$ ,  $R^2 = 0.200$ ,  $P < .001$ ; morphospecies:  $F = 284.67$ ,  $R^2 = 0.567$ ,  $P < .001$ ), and slightly significant differences among replicates ( $F = 4.455$ ,  $R^2 = .020$ ,  $P = .030$ ). Differences between both morphospecies were highly significant in the parent and  $F_1$  offspring generations, but they were more pronounced in the offspring generation (wild-caught parents:  $F = 43.284$ ,  $R^2 = .520$ ,  $P < .001$ ;  $F_1$  offspring:  $F = 307.47$ ,  $R^2 = .637$ ,  $P < .001$ ). Most of the pairwise population comparisons with Hotelling  $T^2$  tests indicated statistically significant differences (Table 1).

Evaluation of the best-supported solutions of model-based clustering is indicated in Figure 6 and the best-supported clusters





**Figure 4.** Two-dimensional morphospace occupation for all specimens in our common garden experiment. A, wild-caught parents; B,  $F_1$  offspring. Both panels are part of the same analysis, with grey circles indicating the total dataset (and thus the position of  $F_1$  offspring in A, and of wild-caught parents in B). The biplot indicates how geometric morphometric variables contribute to the morphospace occupation. Dark grey spheres and separation lines indicate the best-supported solutions of model-based clustering with Gaussian mixture models (see Fig. 6). The recognized clusters correspond well to morphospecies in both generations, although a third group was recognized separating part of the  $F_1$  offspring (i.e. part of replicate 2) of *L. sp. (solidus-like)*.



**Figure 5.** Mean morphospace changes in replicates for our common garden experiment. A, morphospace occupation with arrows indicating transgenerational morphological changes in the means, with error bars representing one standard deviation. All arrows for replicates point in similar directions, with a slightly different trajectory for both *L. sp. (solidus-like)* replicates, however. B, shell shape of *L. sp. (solidus-like)* and *L. sp. (ovum-like)* and transgenerational changes as illustrated by the specimens that most closely resemble the mean morphologies of replicate 1: (a) wild-caught *L. sp. (solidus-like)*, ind. 105; (b) wild-caught *L. sp. (ovum-like)*, ind. 68; (c)  $F_1$  offspring *L. sp. (solidus-like)*, ind. 145; (d)  $F_1$  offspring *L. sp. (ovum-like)*, ind. 36.

are represented with greyscale ellipses and separation lines in Figure 4. For the wild-caught parent generation, we found strong support for a two-group model (EEE was the best supported model, but various other models peaked at a two-group solution; Figs 4A, 6A). Models that do not peak at a two-group

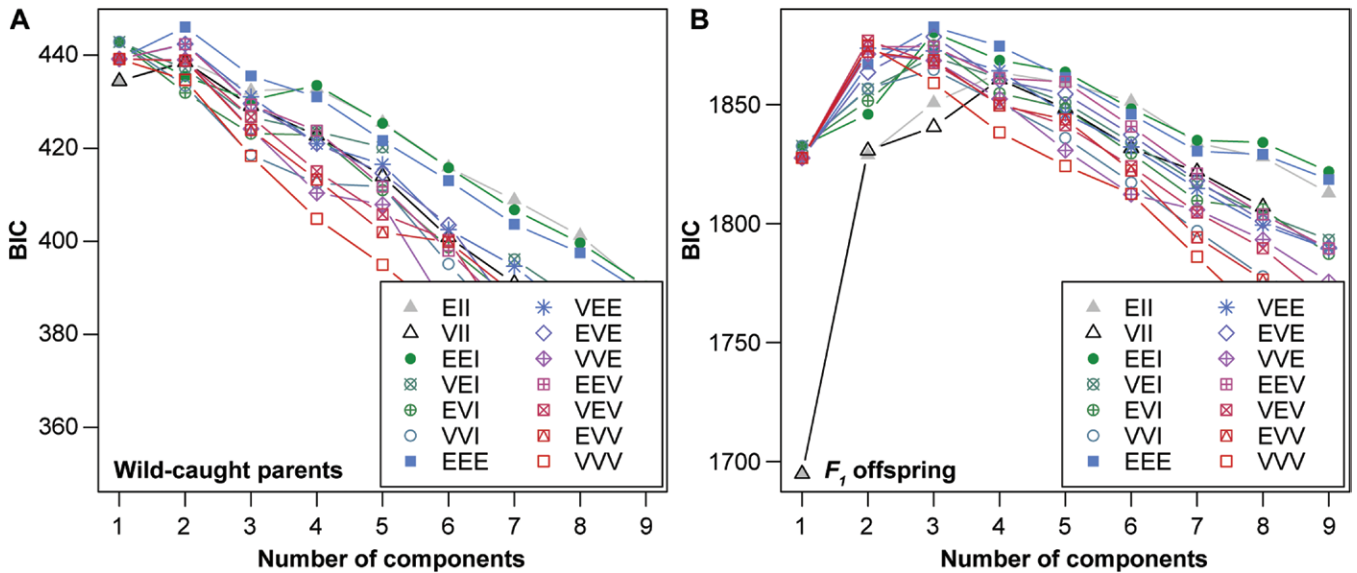
model, such as EEI, suggested a single cluster to be the most likely solution, but such scenarios received substantially smaller BIC support (Fig. 6A). For the  $F_1$  offspring, none of the models suggested a single group; some provided two-group or four-group solutions whereas most preferred a three-group solution



**Table 1.** Results of pairwise morphospecies  $\times$  generation comparisons of geometric shell-morphometric variation with permutational Hotelling  $T^2$  tests (with 10 000 permutations;  $N = 28$ ).

	WP_Lov_r1	WP_Lov_r2	F1_Lov_r1	F1_Lov_r2	WP_Lso_r1	WP_Lso_r2	F1_Lso_r1	F1_Lso_r2
WP_Lov_r1		1.000	<.001	.106	<.001	<.001	<.001	<.001
WP_Lov_r2	1.83		<.001	.566	<.001	<.001	<.001	<.001
F1_Lov_r1	48.73	46.55		.269	<.001	<.001	<.001	<.001
F1_Lov_r2	13.14	8.93	10.17		<.001	<.001	<.001	<.001
WP_Lso_r1	87.06	90.26	38.94	36.27		1.000	<.001	<.001
WP_Lso_r2	87.25	87.83	35.74	32.93	0.09		<.001	<.001
F1_Lso_r1	359.66	413.35	497.95	373.59	60.84	58.03		<.001
F1_Lso_r2	313.19	355.79	362.93	299.24	49.65	47.38	69.74	

Permutational  $T^2$  test statistic and associated Bonferroni-corrected  $P$ -values are provided in the lower and upper triangles, respectively (with significant  $P$ -values in bold). WP = wild-caught parent; F1 =  $F_1$  offspring, Lov = *Lanistes* sp. (*ovum*-like), Lso = *L.* sp. (*solidus*-like); r1 and r2 indicate replicates 1 and 2, respectively.



**Figure 6.** Model support for the various Gaussian mixture models for A, wild-caught parents and B,  $F_1$  offspring in function of the considered number of components, i.e. morphospace clusters. A Bayesian information criterion (BIC) was used to examine the fit of clustering solutions under 14 models of how variance–covariance could be structured in the data. Specifically, these models differ in the distribution of the covariance (spherical, diagonal, ellipsoid) as well as the volume (equal or variable), the shape (equal or variable), and the orientation (none, equal, variable, or along coordinate axes) of within-group variance. The best-supported model for both wild-caught parents and  $F_1$  offspring is EEE, an ellipsoidal model with equal volume, shape, and orientation, and thus with a shared variance–covariance structure for all components/morphospace groups. For more information on models and the differences among them, see [Scrucca et al. \(2016\)](#).

(Fig. 6B). The three-group solution received the highest BIC support and recognized the main split between  $F_1$  offspring of *L.* sp. (*ovum*-like) and *L.* sp. (*solidus*-like), but with a further subdivision of *L.* sp. (*solidus*-like) into two clusters (Figs 4B, 6B). One of these clusters contained specimens of both replicates, but the other only contained specimens of *L.* sp. (*solidus*-like) replicate 2. The best two-group models effectively separated both morphospecies—their solution was identical to the main separation in the three-group model.

## DISCUSSION

**Our experiment is robust despite some confounding factors that are highlighted by analysis of replicates**

Our experiment overall displays highly similar trends among replicates, and therefore it provides robust insights into the

nature of morphological differences between *L.* sp. (*ovum*-like) and *L.* sp. (*solidus*-like) from the northern region of the Malawi Basin, regarding both shell size and shape. Some significant differences are observed between the replicates of the same morphospecies, but they are consistently smaller than differences among generations and among morphospecies. The cause of these differences between replicates is incompletely known, but the overall relatively small parent populations may explain part of these differences for two reasons. As mentioned, the consistently smaller parent populations compared to  $F_1$  offspring populations implies reduced statistical power for tests on parents compared to tests on offspring. Second, parents may have contributed unevenly to the  $F_1$  offspring generation and associated enhanced stochasticity may explain part of the morphological differences among  $F_1$  offspring between replicates of the same morphospecies. Regarding shell size, resampling tests indicated

that differences in sample sizes are responsible for differences in  $F_1$  offspring for *L. sp. (solidus-like)*, but that this issue cannot entirely explain differences in both replicates of  $F_1$  offspring for *L. sp. (ovum-like)*. For this morphospecies, growth was reduced in replicate 1, which contained almost double the number of offspring individuals than replicate 2, so effects of crowding may partly explain the reduced growth in replicate 1 versus replicate 2.

Regarding shell morphology, we observe substantial differences only in the replicates of *L. sp. (solidus-like)*, but these differences were again smaller than the differences among generations and among morphospecies. Nevertheless, model-based clustering on variation in shell shape resulted in the recognition of two clusters for the  $F_1$  offspring of *L. sp. (solidus-like)*. The absence of statistically significant differences for *L. sp. (ovum-like)* indicates that crowding does not have an effect on shell shape, or at least not for levels of crowding similar to those in our experiment. We examined the differences in the  $F_1$  offspring of *L. sp. (solidus-like)* in both replicates using comparisons of Euclidean distances among morphospecies, generations, and replicates directly in the morphospace. These examinations suggest that the observed differences cannot be explained by differences in statistical power alone. Examining the results of model-based clustering for the  $F_1$  offspring of *L. sp. (solidus-like)* in more detail, we observe that one cluster (that with positive values on both nmMDS1 and nmMDS2 in Fig. 4B) contains individuals of both replicates that broadly overlap in morphospace, whereas the other cluster exclusively contains individuals of replicate 2. Given that shell morphology is probably a highly polygenic trait, differences in the genetic constitution of the parent populations, or differences in the contribution of parent genotypes to the offspring populations, may have contributed to the observed morphological differences between replicates. Given our results and how we constituted experimental populations the second explanation is more likely, as it explains both the broad overlap of  $F_1$  offspring from both replicates of *L. sp. (solidus-like)* in part of the morphospace and the enhanced variability in replicate 2 with a subset of specimens extending to another region of the morphospace. As a result of this morphospace extension, our best-supported models of model-based clustering proposed two clusters for *L. sp. (solidus-like)*, but both of these clusters differ substantially in their density of specimens. As this morphospace extension occurs in a direction away from the wild-caught parents and away from the morphospace occupation of *L. sp. (ovum-like)*, it does not interfere with the main questions addressed here. Nevertheless, we have developed a bait set to enrich and sequence the open reading frames of ~2000 genes in *Lanistes* following the workflow of Ortiz-Sepulveda *et al.* (2023), and this can be used in the future together with existing genomic resources (Ip *et al.* 2018, Sun *et al.* 2019) to better understand the genomic underpinnings of shell-morphological differences in *Lanistes*.

#### Intergenerational differences exist and are influenced by allometry

We observed differences between wild-caught parents and  $F_1$  offspring, which may partly relate to differences between our simplified laboratory conditions versus natural environments in the Malawi Basin. Similar differences have been observed

in experiments with the apple snail *Pomacea canaliculata*, which suggest an influence of the environment on shell shape (Estebenet and Martín 2003), but the species also displays allometric changes in shell shape throughout its ontogeny (see Hayes *et al.* 2015). In our experiment we expect that most of our observed differences relate to allometry, as the larger shell size of our wild-caught parents compared to those of  $F_1$  offspring suggests substantial age differences (Fig. 2). Under this hypothesis, shape differences (and thus morphospace distances) should decrease as age (and thus size) differences between parents and offspring decrease, which we observed previously in *Lanistes* from the southern region of the Malawi Basin (Van Bocxlaer *et al.* 2020). Allometric changes in *Lanistes* from the northern region of the Malawi Basin are corroborated by the absence of statistically significant differences in shell morphology in *L. sp. (ovum-like)* between wild-caught parents and the  $F_1$  offspring of replicate 2 (Table 1; Fig. 5). Of all  $F_1$  offspring populations the individuals of *L. sp. (ovum-like)* replicate 2 grew largest, and, as such, these individuals approximate wild-caught parents best in shell size. Further study of wild-caught parents and laboratory-reared offspring at equal sizes may allow us to precisely quantify shape changes between natural and laboratory conditions, but answering the question of whether environment–phenotype correlations among morphospecies of *Lanistes* relate to genetic inheritance versus ecophenotypic plasticity, which we examine in detail below, also provides an answer (see Fig. 1).

#### Shell-morphological differences are genetically inherited

Regarding differences among morphospecies, we observed that *L. sp. (ovum-like)* and *L. sp. (solidus-like)* from the northern region of the Malawi Basin attain similar adult sizes, but that *L. sp. (solidus-like)* grows somewhat more slowly. Differences in shell morphology between wild-caught *L. sp. (ovum-like)* and *L. sp. (solidus-like)* from the northern region of the Malawi Basin persisted in  $F_1$  offspring raised in a common laboratory environment, indicating that these differences are genetically inherited (scenario of Fig. 1A). In *Lanistes* from the southern region of the Malawi Basin, similar experiments have likewise indicated genetical inheritance, but some evidence for ecophenotypic plasticity was also observed (Van Bocxlaer *et al.* 2020). Signals indicative of both genetic inheritance and ecophenotypic plasticity have also been observed in common garden experiments with other ampullariids (Estebenet and Martín 2003), and other caenogastropods (Urabe 2000, Kistner and Dybdahl 2013), but most studies have mainly dealt with intraspecific variation (see Whelan 2021). In contrast, we observed hardly any evidence for ecophenotypic plasticity in *L. sp. (ovum-like)* and *L. sp. (solidus-like)* from the northern region of the Malawi Basin, as shell-morphological differences among morphospecies are proportionally larger in the  $F_1$  offspring than in wild-caught parents. Under the hypothesis of ecophenotypic plasticity, we would have furthermore expected larger changes in shell shape between wild-caught parents and  $F_1$  offspring for *L. sp. (ovum-like)* than for *L. sp. (solidus-like)* because of larger dissimilarity between the natural habitat and experimental conditions, but such a pattern is not what we observed here. In summary, our results indicate that morphological differences between *L. sp. (ovum-like)* and *L. sp. (solidus-like)* from the northern region of the Malawi Basin are mainly or exclusively genetically determined. To our

knowledge, similarly strong evidence for the genetic inheritance of intra- and interspecific variation in shell shape in other caenogastropods currently only exists for the pleurocerid genus *Leptoxis*, although the scarcity of common garden experiments with gastropods may partly explain this lack (Whelan, Johnson and Harris 2015, Whelan 2021). Our result is remarkable given that previous studies of population-genetic differentiation in the northern region of the Malawi Basin based on AFLP indicated the existence of two gene pools that overlap entirely in their morphospace occupation (Van Bocxlaer 2017). Therefore, our common garden experiment suggests that other genetic differences exist among our studied morphospecies, indicating the need for follow-up studies of genetic differentiation with a larger set of molecular markers (see above). If covering a large panel of single nucleotide polymorphisms, such genomic markers would furthermore allow us to study demography and to quantify effective migration rates and introgression among our studied morphospecies (see, e.g., Roux et al. 2016, Fraisse et al. 2020, Ortiz-Sepulveda et al. 2023).

#### Adaptive differences relate to a fitness differential

Our experimental conditions resemble stable, intralacustrine environments rather than the fluctuating conditions of comparatively small satellite waterbodies. Beyond genetic differentiation in shell morphology, we observe that *L. sp. (solidus-like)*, the morphospecies that occupies stable, intralacustrine environments, displays significantly higher fecundity than *L. sp. (ovum-like)* in our experiment. These results indicate a relationship between genetic differences in shell morphology and fecundity presumably due to larger genotype–environment mismatches between the natural habitat and our experimental conditions for *L. sp. (ovum-like)* than for *L. sp. (solidus-like)*. Therefore, they suggest that the observed differences are either adaptive, or associated with adaptation in another, unmeasured trait (see Estebenet and Martín 2003). Interestingly, similar results on fecundity were obtained in a common garden experiment with *Lanistes* morphospecies from the southern region of the Malawi Basin (Van Bocxlaer et al. 2020), so jointly these results support the occurrence of local adaptation, but fully reciprocal transplant experiments would be required to confirm this issue. Additionally, the use of genome-wide genetic markers could allow the study of local adaptation using population genetics to gain understanding in spatial patterns and drivers of differentiation, for example by comparing patterns at genomic sites that evolve neutrally compared to sites under selection (Tiffin and Ross-Ibarra 2014, Sunde et al. 2022).

Various other observations on *Lanistes* from the northern region of the Malawi Basin allow us to link morphospecies and the genetic nature of shell-morphological differences to differential fitness in stable, intralacustrine conditions versus fluctuating conditions characterizing satellite waterbodies. Fluctuations occur mainly in temperature, dissolved oxygen concentrations, and the risk of desiccation, although other environmental variables may differ too, notably regarding vegetation and the amount and type of detritus available. Under unpredictable environmental conditions and/or stronger seasonality, as occur in satellite waterbodies, faster growth and maturation may be advantageous. For example, at smaller size snails have a larger surface-to-volume ratio, which would result in faster heating

and more limited capacities of thermoregulation (Schweizer et al. 2019). Such factors could explain the faster growth that we observed in *L. sp. (ovum-like)* compared to *L. sp. (solidus-like)* from the northern region of the Malawi Basin. Interestingly, no such growth differences have been observed in *L. solidus* and *L. sp. (ovum-like)* from the southern region of the Malawi Basin (Van Bocxlaer et al. 2020).

The observed shell shape differences among *L. sp. (ovum-like)* and *L. sp. (solidus-like)* mainly reflect differences in the height of the spire, the size of the aperture relative to the total shell size, and whether the umbilicus is open or closed (Figs 3–5). These differences have been interpreted as adaptations to wave action and differences in the types of predators among habitats. The thick, short-spined shells with narrow or closed umbilicus of *L. sp. (solidus-like)* can resist more mechanical stress than the thinner, higher spired shells with wide open umbilicus of *L. sp. (ovum-like)*, and would thus be advantageous in intralacustrine, nearshore habitats with high water energy (Vermeij 1982, Louda et al. 1984, Berthold 1990a). Furthermore, predation in such intralacustrine habitats would occur mainly by cichlids and crabs, against which shell-thickening is a recognized defence mechanism (Vermeij 1982), whereas in satellite waterbodies birds are the main predators. Furthermore, wave action is reduced in satellite waterbodies, among others by more abundant shelter-providing fringing vegetation. A smaller aperture (rather than a thicker shell) may reduce the success of attempts of predation by birds and could limit desiccation stress in these latter environments.

An additional interesting observation in this respect is that our stable experimental conditions did not induce spontaneous reproduction in *L. sp. (ovum-like)* from the northern region of the Malawi Basin, but that exposure to a heat wave was required. We likewise subjected *L. sp. (solidus-like)* to heat waves, but it did not induce reproduction in this morphospecies. In this context, it is noteworthy that *L. sp. (ovum-like)* from the southern region, which occurs primarily in satellite waterbodies too, does not require exposure to a heat wave to induce reproduction (Van Bocxlaer et al. 2020), but we cannot currently exclude that such exposure may enhance reproduction. The differential cues for reproduction between *L. sp. (ovum-like)* and *L. sp. (solidus-like)* from the northern region of the Malawi Basin could result in assortative mating when both morphospecies become sympatric, either by flooding of satellite waterbodies or displacement of *L. sp. (ovum-like)* individuals to the lake by flash floods. In both cases, the environmental fluctuations that *L. sp. (ovum-like)* usually experiences would be buffered, which would hence obliterate the environmental cue that we used to trigger reproduction in *L. sp. (ovum-like)*. Nevertheless, it is conceivable that other mechanisms may exist in nature that could break down environmentally induced assortative mating. A candidate mechanism is our observation that, once some individuals in a population start reproducing, other individuals are triggered to reproduce too, perhaps due to chemical cues, because the mechanism seems functional upon placing a nonreproducing population in flow-through with a reproducing population (although we do not currently have all the required quantitative data to formally test this hypothesis). We hypothesize that a strong driver to synchronize reproduction in *Lanistes* relates to juvenile behaviour. Eggs are deposited in a nutritious gelatinous envelope that serves as food



to recently hatched juveniles, with juveniles feeding preferentially on this envelope, other eggs, and proximate egg clutches. Therefore, the late deposition of egg clutches strongly reduces fecundity due to juvenile egg consumption. If this behaviour occurs in the wild, it would present a strong fitness cost to mothers that reproduce late, but to what extent and at what spatial scales it impacts natural populations are uncertain.

## CONCLUSION

We observed differences in fitness, growth, and shell morphology upon subjecting two morphospecies, *L. sp.* (*ovum*-like) and *L. sp.* (*solidus*-like), from the northern region of the Malawi Basin to common garden experiments in the laboratory. The persistence of differences that were observed in the wild under controlled conditions supports the hypothesis that these differences are genetically inherited and potentially adaptive in light of associated fitness differences. Therefore, the environment–phenotype correlations that we observe in *Lanistes* from the northern region of the Malawi Basin, together with the genetic inheritance of shell morphology in morphospecies from the southern region of the Malawi Basin (Van Bocxlaer *et al.* 2020) and the documentation of a pattern of isolation-by-adaptation within (but not between) both the northern and southern regions of the Malawi Basin (Van Bocxlaer 2017) are all indicative of adaptive divergence among morphospecies within regions. Our results, therewith, strongly suggest that the ongoing *Lanistes* radiation is adaptive, although neutral divergence and parallel evolution at both sides of the migration barrier that separates the northern and southern regions are also important features of this radiation.

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## CREDIT STATEMENT

Bert Van Bocxlaer (conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing—original draft, writing—review & editing, visualization, supervision, project administration, funding acquisition); Alexis Y. Dollion (investigation, formal analysis, writing—review & editing); Claudia M. Ortiz Sepulveda (investigation, formal analysis, writing—review & editing); Christophe Calarnou (investigation, formal analysis); Rémi Habert (investigation, formal analysis); Grant Pawindo (investigation, resources), and Xavier Vekemans (resources, writing—review & editing, funding acquisition). All authors read and approved the final manuscript.

## CONFLICT OF INTEREST

We are not aware of any competing interests.

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## DATA AVAILABILITY STATEMENT

The data underlying this article are available in its online [Supplementary Material](#).

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