

# SPECIES-SPECIFIC DIFFERENCES IN ADAPTIVE PHENOTYPIC PLASTICITY IN AN ECOLOGICALLY RELEVANT TROPHIC TRAIT: HYPERTROPHIC LIPS IN MIDAS CICHLID FISHES

Gonzalo Machado-Schiaffino,<sup>1,\*</sup> Frederico Henning,<sup>1,\*</sup> and Axel Meyer<sup>1,2</sup>

<sup>1</sup>Chair of Zoology and Evolutionary Biology, Department of Biology, University of Konstanz, Universitaetsstrasse 10, 78457, Konstanz, Germany

<sup>2</sup>E-mail: axel.meyer@uni-konstanz.de

Received September 6, 2013

Accepted January 14, 2014

The spectacular species richness of cichlids and their diversity in morphology, coloration, and behavior have made them an ideal model for the study of speciation and adaptive evolution. Hypertrophic lips evolved repeatedly and independently in African and Neotropical cichlid radiations. Cichlids with hypertrophic lips forage predominantly in rocky crevices and it has been hypothesized that mechanical stress caused by friction could result in larger lips through phenotypic plasticity. To test the influence of the environment on the size and development of lips, we conducted a series of breeding and feeding experiments on Midas cichlids. Full-sibs of *Amphilophus labiatus* (thick-lipped) and *Amphilophus citrinellus* (thin-lipped) each were split into a control group which was fed food from the water column and a treatment group whose food was fixed to substrates. We found strong evidence for phenotypic plasticity on lip area in the thick-lipped species, but not in the thin-lipped species. Intermediate phenotypic values were observed in hybrids from thick- and thin-lipped species reared under "control" conditions. Thus, both a genetic, but also a phenotypic plastic component is involved in the development of hypertrophic lips in Neotropical cichlids. Moreover, species-specific adaptive phenotypic plasticity was found, suggesting that plasticity is selected for in recent thick-lipped species.

**KEY WORDS:** Adaptive phenotypic plasticity, *Amphilophus labiatus*, *Amphilophus citrinellus*, Hypertrophic lips, Midas cichlids, Reaction norm.

Phenotypic plasticity, the ability of a single genotype to produce multiple phenotypes in response to variation in the environment, has received renewed attention from the scientific community in last decade (DeWitt et al. 1998; Pigliucci and Murren 2003; West-Eberhard 2003; Grether 2005; Ghalambor et al. 2007; Lande 2009). It has been suggested that phenotypic plasticity could play a key role in evolution by promoting: (i) the origin of novel phenotypes, (ii) divergence among populations and species, (iii) the formation of new species, and (iv) adaptive radiation (reviewed in Pfennig et al. 2010).

Adaptive phenotypic plasticity—the potential for an organism to produce a range of different, but adaptive, multiple phenotypes—might be crucially beneficial for the colonization of new, or variable environments such as those found at the initial steps of ecological speciation. It has been hypothesized that plasticity facilitates adaptive peak shifts, by placing populations close enough to a new adaptive peak to allow for the subsequent action of directional selection (Ghalambor et al. 2007). When natural selection acts on genetic variants affecting the trait in the same direction as environmentally induced plasticity it might become genetically determined and canalized in a process known as genetic assimilation (Waddington 1942, 1953; Pigliucci and Murren

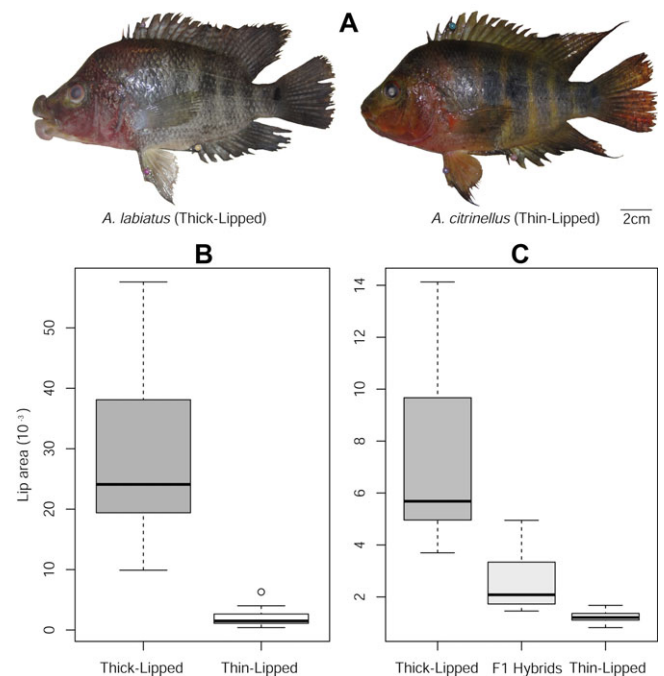
\*These authors contributed equally to this work.

2003; Price et al. 2003; West-Eberhard 2003). Furthermore, it has been proposed that the cost of plasticity (DeWitt et al. 1998) may lead to genetic assimilation even under moderately heterogeneous environmental conditions (Mayley 1996).

Phenotypic plasticity, followed by genetic assimilation, also has been suggested to be an important mechanism during the formation of adaptive radiations (Losos et al. 2000). The spectacular species richness of cichlid fishes and their famous phenotypic diversity have made them a well-known model for the study of speciation and adaptive radiation (Fryer and Iles 1972; Meyer 1993; Salzburger and Meyer 2004). Interestingly, distantly related species from different lakes exhibit remarkable phenotypic resemblance, converging on several traits such as coloration, body shape, and trophic morphology (Kocher et al. 1993; Ruber et al. 1999; Stiassny and Meyer 1999; Albertson and Kocher 2006), and one of the most remarkable cases of convergence among cichlid lineages is the evolution of hypertrophic lips.

Thick lips evolved repeatedly and independently in African (e.g. *Haplochromis chilotes* in Lake Victoria, *Abactochromis labrosus* in Lake Malawi and *Lobochilotes labiatus* in Lake Tanganyika) and Neotropical cichlid radiations (e.g. *Crenicichla iguassuensis* in Iguazu River and *A. labiatus* from Lake Nicaragua, Fig. 1A). The fact that thick lips evolved repeatedly in parallel strongly suggests that they are an adaptation (Losos 2011), and evolved probably in response to similar selective pressures associated with a particular type of foraging behaviour in rocky areas. It has been proposed that hypertrophic lips enhance a fish's ability to forage in rocky substrates by facilitating the access to crustacean and fish larvae that hide between rocks (Barlow and Munsey 1976; Arnegard and Snoeks 2001; Oliver and Arnegard 2010). Thick-lipped fish tend to have slender, narrow, and pointed heads that allow them to access to rocky crevices and capture their prey. The lips serve both as a seal as well as a "bumper" that protect the mouths of the fish against injury by the sharp-edged rock in volcanic lakes (Greenwood 1974). Different trophic niche exploitation has been reported between thick- and thin-lipped species both in Neotropical and African lakes (Elmer et al. 2010; Colombo et al. 2013; Manousaki et al. 2013), where thick-lipped species preferentially feed on benthic crustaceans such as crabs and shrimps which are more abundant in rocky areas. Within the Midas species complex, thick-lipped species are present in the ancestral great Lakes Managua and Nicaragua (*A. labiatus*) as well as in two crater lakes (Lake Apoyeque and Masaya) that were initially colonized from the large lakes (Barluenga and Meyer 2010). Notably, thick-lipped fish coexist with the "ancestral" and geographically more widespread thin-lipped species (*A. citrinellus*) in all lakes of Nicaragua.

Although the hypertrophied lips seem to play an adaptive role in foraging in rocky areas in nature and are a key taxonomic trait, it has been reported that thick-lipped cichlid species



**Figure 1.** (A) Photographs of wild-caught thin-lipped (*A. citrinellus*) and thick-lipped (*A. labiatus*) from lake Nicaragua. (B) Phenotypic distribution of lip areas for both species from a natural population (Lake Nicaragua). (C) Genetic component. Intra- and inter-specific F<sub>1</sub> crosses. Boxplots show the median and interquartile ranges trait values (protuded lip area normalized by body area  $\times 10^{-3}$ ). *Amphilophus labiatus* (thick-lipped) and *A. citrinellus* (thin-lipped) are shown in gray and white, respectively. Notice that the scales on the abscissa are different, the standardized lip area in wild thick-lipped fish is bigger than in laboratory fish.

lose, or at least reduce, their fleshy lips in captivity (Barlow and Munsey 1976). This suggests a phenotypically plastic component on this character, however, this has not been properly tested yet. In the present study, through a combination of different intra- and interspecific crosses, combined with forced feeding experiments (inducing mechanical stress on lips), we test the role of phenotypic plasticity in hypertrophic lips in thin- and thick-lipped sister species. Under a scenario of adaptive phenotypic plasticity in hypertrophic lips, higher morphologic variation (plasticity) is expected in the thick-lipped species.

## Materials and Methods

### LIP MEASUREMENTS

Two independent sets of pictures per individual were taken for a batch of 50 individuals to evaluate the repeatability of different measurements related to hypertrophic lip phenotype (protuded lip area and lip length) and body size (standard length and body area). All measurements were taken from fish anaesthetized with MS-222 (Sigma) in standardized photographs taken from the

dorsal and lateral views (Supporting Information Fig. S1). Measurements were performed using ImageJ software (Schneider et al. 2012).

Repeatability was higher than 95% for protruded lip and body area indicating that the error in estimating the different phenotypes is very low. This measurement (protruded lip area normalized by body area) was found to reflect the species' phenotypic differences and to be more repeatable than lip length (measured from the dorsal view and normalized by standard length). Hereafter, we refer to lip area as the protruded lip area normalized by body area and all values are expressed in ratios.

### LIP SIZE DISTRIBUTION IN FIELD COLLECTED SPECIES

The differences in the head morphology particularly regarding the feeding apparatus of the thin-lipped *A. citrinellus* and the thick-lipped *A. labiatus* are quite evident (see Fig. 1A). *Amphilophus labiatus*, as the name suggests, has more protruded lips, a narrower and more pointed head. Standardized photographs of wild caught individuals from each species were taken to quantify these differences. Fifty individuals per species were collected in Lake Nicaragua in March 2013.

### ONTOGENETIC EFFECT ON HYPERTROPHIC LIPS

To investigate whether allometric effects are different during the ontogeny for thin- and thick-lipped species, an independent experiment was performed including a group of 20 *A. citrinellus* and 20 *A. labiatus* that were kept in separate tanks under similar conditions (density, food, temperature, salinity, pH, light/darkness hours, etc.) in the Animal Research Facility at the University of Konstanz. Standardized photographs were taken every 2.5 months for a total period of 10 months.

### FORCED FEEDING EXPERIMENT

One full sibfamily ( $n = 50$ ) from a laboratory stock of *A. citrinellus* derived from individuals collected in the great Lake Nicaragua in 2007 and one from *A. labiatus* ( $n = 50$ ), derived from individuals collected in the crater Lake Masaya in 2005, were split randomly at the age of two months, to generate the control (C) and treatment (T) groups for each species. These four groups were raised each in a 50 L tank for two months, then transferred to 400 L tanks for another four months. Control fish were fed daily *Artemia nauplii* "floating" in the water for the first four months, followed by mosquito larvae for two months. Treatment fish were fed daily with the same amount of *Artemia nauplii*, but the food was attached (by drying for 30 min at 60°C) to a thin mesh for the first two months. In the following two months the Artemia diet of the treatment group was dried to medium grain sandpaper—to simulate the abrasive surface of volcanic rock surfaces. Finally,

fish in the treatment group were fed mosquito larvae that had been dried on volcanic rocks. The control and treatment groups were otherwise cared for identically. All experiments were approved by the German authorities (Permit of the Regierungspräsidium Freiburg, Abteilung Landwirtschaft, number 35–9185.81/G-13/59).

### GENETIC COMPONENT OF LIP TRAIT

To test whether the presence of hypertrophic lips has a genetic component, intra- and interspecific crosses were performed for thick- and thin-lipped Midas species. All crosses yielded > 100  $F_1$ s, of which 50 were reared to 24 months in 2000 L tanks. All fish were kept under the same standardized conditions and were photographed at 22 months of age.

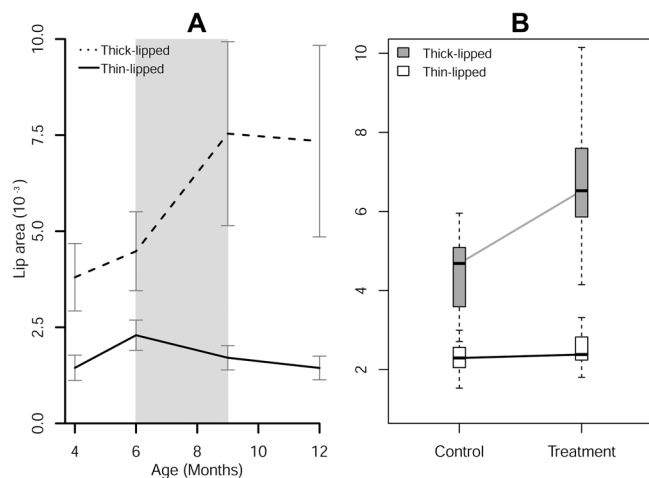
## Results

The distribution of standardized lip area values in field-collected samples of thin- and thick-lipped Midas cichlids in the great Lake Managua are completely nonoverlapping and the within-species distributions differ as well (Fig. 1B). Not only is the median trait value higher, but also the variation and overall range is larger in *A. labiatus* (Fig. 1B). The hypertrophied lips of captive bred thick-lipped species were much less pronounced than those found in wild-caught specimens, suggesting a strong phenotypically plastic aspect to lip development (Fig. 1C).

In the study of the ontogenetic effect of hypertrophic lip experiment, two main differences were observed when comparing thick- and thin-lipped Midas cichlids species bred in captivity under the same conditions. First, thick-lipped *A. labiatus* already had lips that were roughly twice as large, in comparison with *A. citrinellus*, by the time of the first measurement (four-month-old fish). Second, this difference increased at a precise time point. The lip size of *A. labiatus* increased particularly strongly between six and nine months. However, the thin-lipped species *A. citrinellus* did not change the shape of its lips and stayed on its isometric ontogenetic trajectory (Fig. 2A).

We found strong evidence for mechanical-stress induced plasticity in lip area after six months of treatment. The feeding experiment induced differential lip growth in thick-lipped species. However, we did not find evidence for a phenotypic response in the treatment group for thin-lipped species (Fig. 2B). The species/treatment interaction was highly significant indicating that the response to treatment varies between the species (Table 1).

Finally, a clear nonrandom and intermediate distribution of lip area was found in the  $F_1$  hybrids reared under "control" conditions, which might indicate that the presence hypertrophic lips has also a genetic component (Fig. 1C).



**Figure 2.** Lip area standardized by body area ( $\times 10^{-3}$ ). (A) Ontogenetic trajectories of hypertrophic lips measured every 2.5 months for a period of ten months. (B) Phenotypic distribution after six months of forced feeding experiment (eight months old fish).

**Table 1.** Statistical analysis of phenotypic plasticity of lip area in Midas cichlids after 6 months of forced feeding. The table shows the results of a linear model with interaction considering “lip area” as response variable and “treatment group” and “species” as explanatory variables.

	Estimate	Std. error	<i>t</i> value	<i>P</i>
Treatment	0.40	0.08357	4.81	***
Species	-0.66	0.08992	-7.31	***
Interaction	-0.36	0.11930	-3.00	**

## Discussion

Variation in lip size in Midas cichlids clearly has a strong environmental phenotypically plastic component that affect both the size and the response to environmental differences. The plastic response is in the same direction as selection for foraging performance, suggesting that it is adaptive for rock-dwelling cichlids. Phenotypic plasticity could only be detected in the thick-lipped species (feeding experiment). Potentially genetic species-specific lip growth variation is expressed in two life stages, one before four months and another between six and nine months of age (ontogenetic effect on lips). Taken together, these results suggest that differences exist both in terms of lip size as well as plastic response between these two sisters species. This raises the exciting possibility that plasticity has been selected for. Thus, our findings of a genetic basis and of phenotypic plasticity in hypertrophic lips in Midas cichlids agree with the suggested importance of both divergent natural selection and adaptive phenotypic plasticity acting on ecologically relevant traits in adaptive radiations (Schluter 2000; Pfennig et al. 2010).

The species-specific differences appear to affect different aspects of the phenotype. Although thick-lipped fish already start with higher phenotypic values, the difference in relation to thin-lipped fish increases at a particular life stage (Fig. 2A). An interesting hypothesis is that the period between six and nine months is when hypertrophic lips have a strong adaptive value. This could occur either by shifts in diet, habitat or simply the fact that small fish are not constrained by size to forage in rock crevices. The hypothesis that the period of increased growth has been brought about by selection merits further investigation.

The existence of a period of accelerated lip growth should be also taken into account in future studies of gene expression associated with hypertrophic lips. Two studies have investigated gene expression of hypertrophic lips in wild caught specimens, reporting parallel changes in gene expression across lakes (Manousaki et al. 2013) and continents (Colombo et al. 2013). However, probably due to the large variation in the age and condition of wild-caught samples as well as the different methodological approaches, only a few overlapping set of genes between both studies was found (e.g. *gtpase* *imap* family member, *GIMAP*).

Although the results of the breeding experiment and captive rearing suggest a genetic component for species differences in lip size, both the variation and the lips were smaller than those observed in natural populations, suggesting a strong effect of phenotypic plasticity in this trait (Fig. 1). It had been speculated before whether mechanically induced phenotypic plasticity could affect this trait (Barlow and Munsey 1976), but this is the first time that a formal experiment is performed to test that hypothesis.

We did indeed find evidence of plastic response and it was in the expected direction (increase in lip area) potentially favored by natural selection for foraging performance in rock-crevices. Therefore, the phenotypically plastic response can be considered adaptive. Plasticity seems to play a much greater role in the thick-lipped compared to thin-lipped Midas cichlids, opening the possibility that selection has also acted on genes that affect plastic response. An alternative interpretation is that selection favored the increase of an already plastic tissue. In this case, plastic response might not have been directly selected for. This would predict that individuals with higher initial trait values would have steeper slopes. Unfortunately, these two scenarios cannot be disentangled with the current data.

Adaptive phenotypic plasticity has been previously observed in other ecologically relevant traits in cichlids and might play an important role in the propensity of cichlids to undergo rapid adaptive radiation. Pharyngeal jaw morphology has been shown to respond plastically in both African (Greenwood 1973; Gunter et al. 2013) and Neotropical cichlids (Meyer 1987; Muschick et al. 2011). Midas cichlids are a young adaptive radiation where speciation can still be observed at different stages of completion (Barluenga et al. 2006; Elmer and Meyer 2011; Kautt et al. 2012).



It has been recently suggested that adaptive phenotypic plasticity in pharyngeal jaw of *A. citrinellus* could play a crucial role in ecological speciation and adaptive radiation in the repeated *Amphilophus* crater-lake radiations (Muschick et al. 2011). The fact that plastic response was not detected in the “ancestral” species from lake Nicaragua (*A. citrinellus*) and a significant response was found in the recently colonized (derived) crater lake Masaya specimens, may suggest a potential role of plasticity in the colonization and adaptation to a new environment (crater lakes). Even though further studies including more replicates should be performed, these results are in agreement with the model of genetic accommodation, where an originally environmental insensitive phenotype is converted into an environmentally sensitive one (e.g. West-Eberhard 2003; Suzuki and Nijhout 2006, Rajakumar et al. 2012). The difference in lip morphology between captive-bred and wild-caught specimens of the more anciently diverged Lake Victorian thick-lipped cichlid *Haplochromis chilotes* seems to be much smaller than in Midas cichlids, which is suggestive of a weaker plastic component (A. Meyer, unpubl. data). Further studies including more species should be conducted to analyze in more detail whether the plastic response is decreased in more anciently diverged (more differentiated) cichlid species than in younger species.

Several lines of evidence indicate that the phenotypic differences between thick- and thin-lipped Midas cichlids are the result of disruptive selection (Elmer et al. 2010). These include the strong bimodality observed in the field-collected specimens (Fig. 1B), known ecological differences between the thick- and thin-lipped species (Barlow and Munsey 1976; Colombo et al. 2013; Manousaki et al. 2013) and the function of parallel phenotypes from African cichlids (Fryer and Iles 1972; Arnegard et al. 1999). There is also evidence that the number of genes that are differentially expressed between thin- and thick-lipped cichlids increases with the divergence time of thin- and thick-lipped species (Manousaki et al. 2013). This could be seen as an indication of the effects of continued divergent selection. The persistence of phenotypic differences in a common environment after several generations (Barlow and Munsey 1976) and the intermediate values of  $F_1$  hybrids reared under “control” conditions suggest that the presence of hypertrophic lips has a genetic component. Our results argue for a multivariate genetic basis with a great degree of environmental variance and potentially GxE interaction. The pattern of codominance that was found is compatible with several genetic architectures (e.g. the presence of a major gene or the additive effect polygenes). Formal analyses of heritability, analysis of plasticity in the hybrids and genetic mapping experiments are necessary to unravel the genetic architecture of the environmental responsiveness of this trait.

In summary, we detected species-specific differences in plastic components of the ecologically crucial hypertrophic lips in

two different species of the Midas cichlid species radiation of Nicaragua. Phenotypic plasticity was detected only in the thick-lipped species *A. labiatus* and not in its sister species, the thin-lipped *A. citrinellus*. The plastic response occurred in same direction as putative selection, suggesting that the phenotypic plasticity is adaptive. This suggests that putative genetic differences in plastic response exist between these species and opens the possibility that plasticity has been selected for. Moreover, we also identified a particular ontogenetic stage where these differences are likely to be expressed. All these differences probably affect foraging performance in divergent ecological niches and might play a crucial role in the early stages of cichlid adaptive radiations.

#### ACKNOWLEDGMENTS

This research was funded by Alexander von Humboldt fellowship and Deutsche Forschungsgemeinschaft (DFG MA6144/1–1) to GMS, the Brazilian National Council for Scientific and Technological Development (CNPq)/German Academic Exchange Program (DAAD) cooperation to FH and grants of the DFG and European Research Council (ERC advanced grant “GenAdap” 29700) to AM. We thank MARENA (Ministerio de Ambiente y Recursos Naturales, Nicaragua) for sample collection permits, M. Schmidt for technical support and Dr. J. Torres-Dowdall for fruitful discussions. We also thank Dr. Ehab Abouheif and two anonymous reviewers for useful comments that greatly improved this manuscript.

#### DATA ARCHIVING

The doi for our data is 10.5061/dryad.ht62k.

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Associate Editor: E. Abouheif

### Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

**Figure S1.** Lateral and dorsal view of thick-lipped (*A. labiatus*) showing how the measures were taken in this study.