Ecological and Evolutionary Consequences of
Color- and Trophic Polymorphisms
in Cichlid Fishes

DISSERTATION

zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften, Dr. rer. nat.

vorgelegt von

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Fachbereich Biologie

Konstanz, 2013

Konstanzer Online-Publikations-System (KOPS)
URL: http://nbn-resolving.de/urn:nbn:de:bsz:352-253367
Acknowledgements

I would like to thank Prof. Axel Meyer, for opening me the door into the world of evolutionary research on cichlids. I have always encountered a very stimulating atmosphere during my time in the Meyer lab, which encouraged the exchange of scientific ideas and resulted in plenty of promising research ideas. I profited a lot from the generous opportunities to study cichlids in their natural habitats, both from the Old World but also from Neotropical crater lakes. The combined approach of field and laboratory work was very appealing to me. In this spirit, I thank all the Meyer lab members for being so supportive throughout the years in various regards and for the international atmosphere in all its facets. Similar, I am grateful for my enrollment in the International Max Planck Research School (IMPRS) for Organismal Biology and in particular I thank its coordinator Dr. Daniel Piechowski for his efforts in maintaining this platform of scientific idea exchange and for offering such great workshops. I kindly thank Dr. Frederico Henning, Dr. Carmelo Fruciano and Andreas Haerer for their comments on this thesis.

A special “Thank you!” section is reserved for Dr. Kathryn Elmer (University of Glasgow, UK) for her continuous and thorough mentoring during five years of Midas cichlid research. I substantially benefited from her critical advice and her endorsement in the conduction and completion of my projects.

Thanks to Prof. Christian Sturmbauer (University of Graz, Austria) for his continuous involvement as a member of my PhD advisory committee. His generous support during the Lake Tanganyika field trip (2010) enabled me to conduct the research line dealing with scale-eating cichlids.

The last and most important paragraph is reserved for those special contributors from the private side of life that have always endorsed and motivated me to hold on the track, especially when exposed to adverse conditions: My family, friends, and Claudi in particular.
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Abstract

How the exuberant diversity on our planet arose is still a question of central interest in biology. Rapidly diversifying species complexes that experience adaptive radiation have always been prime study targets to infer the underlying mechanisms of divergence. Young species-rich systems that display ongoing diversification in multiple replicates are of particular relevance, because different stages of divergence are likely to be found, ranging from incipient speciation to stable species assemblages consisting of multiple reproductively isolated species. In that framework, various studies focusing on phenotypic variation, on the interaction of phenotype and environment, and consequently of adaptation to different environments have produced manifold insights into the ecological and evolutionary processes leading to diversification. The presence of discontinuous variation in trophic characters or in coloration has by many investigators not only been regarded as an efficient mechanism to achieve a relaxation of intraspecific competition, but also as a signal of ongoing diversification processes.

This doctoral thesis entitled “Ecological and Evolutionary Consequences of Color- and Trophic Polymorphisms in Cichlid Fishes” is aimed at advancing the still limited understanding of the underlying processes leading to the evolution and maintenance of biological diversity. With more than 2,000 described species, cichlid fishes are one of nature’s most biologically diverse groups. Eco-morphologically similar species and color morphs can be observed among independent lineages. Consequently, cichlids are an excellent model for studying the biological, evolutionary, and ecological factors that promote biodiversity and the genesis of new species. The central line of research being addressed throughout this thesis investigates ecological consequences of trophic and color polymorphisms and assesses their maintenance as well as their putative role in lineage diversification in two study systems of cichlid fishes: Midas cichlids from Nicaragua and *Perissodus microlepis*, a scale-eating cichlid from Lake Tanganyika.

Midas cichlids notably occur in the Nicaraguan great lakes and in some small and isolated crater lakes that have been colonized from the great lakes. In some crater lakes Midas cichlids have further diversified and new species have evolved within short time periods, making them prone to study diversification processes. Also, the crater lake populations are often considered being “evolutionary replicates” or “independent laboratories of evolution”. In several crater lakes diversification takes place along the
benthic-limnetic axis and most populations are also color-polymorphic.

The most comprehensive project focuses on the gold/dark color polymorphism in the Midas cichlid species complex (Amphilophus citrinellus spp.). Using a comparative approach that bases on extensive field collections of Midas cichlids, a substantial degree of ecological differentiation between color morphs is revealed. The differentiation is consistent throughout the species complex. From a common garden approach it is inferred, that eco-morphological differentiation between color morphs in the wild is likely partly genetically determined. This is an innovative finding in adaptive radiation research. The correlation of ecology and coloration presumably contributes to the maintenance of this color polymorphism over time through niche partitioning and additionally exhibits great potential to facilitate sympatric diversification processes, given the reported color assortative mating. In a subproject related to coloration, color differential predation by a major Midas cichlid predator (Parachromis managuensis) was revealed using color variants of the common Goldfish that are similar to gold and dark Midas cichlid color morphs. A highly increased attack risk was observed for the Goldfish variant that is similar to the Midas cichlid gold morph. The putative role of color differential predation in maintaining the color polymorphism throughout the evolutionary replicates is discussed. Another project aims at detecting intraspecific diversification along the benthic-limnetic axis in one of the youngest crater lakes of Nicaragua. In each of two older crater lakes multiple Midas cichlid species can be found, that are characteristically diverse along the benthic-limnetic axis. Substantial variability and individual specialization was found in this young Midas cichlid population. This may hint at the incipient evolution of a trophic resource polymorphism and possibly at imminent diversification along the benthic-limnetic axis.

The second case study involved the scale eating cichlid Perissodus microlepis from Lake Tanganyika that is famous for its asymmetric mouth morphology. In some textbooks this species is referred to as an example of a stunning trophic specialization, and of the role of negative frequency-dependent selection in the maintenance of an intraspecific trophic polymorphism. Perissodus microlepis attacks its prey mostly from behind and snatches scales from the prey fish flanks. It is assumed that through the possession of asymmetric mouth morphologies scale-eating from the prey fish flanks is facilitated. So far, this trophic polymorphism has mainly been described as a dimorphic trait, i.e. left (L-morph) or right (R-morph) with no intermediate morphologies. It is thought that L-morphs prefer to attack the right flanks of their prey fish whereas R-
Abstract

morphs rather attack the left prey fish flanks. The maintenance of this polymorphism is thought to be achieved because the prey fish would be more alert against the relatively more often attacked body flank. Hence the common scale-eater morph would suffer a disadvantage, and consequently the rare morph will be favored and eventually increase in frequency. Mouth asymmetry in this species is thought to be determined by two alleles at a single locus, (R is dominant over L and R is homozygous lethal). The pattern of disassortative mating between mouth morphs has been invoked to compensate the reported lethality of the individuals carrying two copies of the dominant R-allele.

One project contributing to this thesis is aimed at shedding light on a recently developing controversial debate, that relates to the distribution of morphological variation (discrete vs. continuous) and to the mating pattern (disassortative vs. random) of this species. Mouth asymmetry in an extensive sample of wild-caught specimens was quantitatively assessed and the mating pattern was reinvestigated. In contrast to previous reports continuously distributed mouth morphologies were found in several populations and random mating was observed. These findings are concordant with that of other recent studies and question the original claims that have been outlined above. In particular they hint at significant environmental contributions to the shaping of morphological laterality in this species. However, given the large geographic distances between our sampling sites and those of previous studies, that have initially coined this textbook example, it is still conceivable that geographic aspects play a role. Additional aspects come from a behavioral perspective: Using wild-caught adult scale-eaters and their natural prey, we predicted the direction of lateralized attack behavior from morphology in the field. In the laboratory, strong behavioral lateralization was found in at most weakly asymmetric laboratory-raised juvenile fish, with respect to morphology. This suggests that morphological asymmetry might be governed by behavioral handedness in this species, somewhat underlining the role of phenotypic plasticity in bringing about morphological asymmetry in this species, that has recently been invoked in the controversial debate surrounding this system.

Overall, this thesis reveals various patterns of ecological diversification based on trophic- and color polymorphisms in cichlid fishes and describes the dynamics of maintenance and the putative role of such polymorphisms in lineage diversification, therefore adding to the understanding of phenotypic evolution in the biodiversity of life.
Zusammenfassung


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Ein weiteres Forschungsvorhaben bezieht sich auf den schuppenfressenden Buntbarsch *Perissodus microlepis* aus dem Tanganjikasee in Afrika. Diese ökologisch erstaunlich spezialisierte Art ist für ihre asymmetrischen Mäuler bekannt und wird in einigen Lehrbüchern als ein Beispiel für die Rolle von negativ-häufigkeitsabhängiger Selektion in der Aufrechterhaltung eines innerartlichen trophischen Polymorphismus herangezogen. Die asymmetrischen Mäuler sind vermutlich vorteilhaft für die effiziente Ausübung ihres natürlichen Nahrungsaufnahmeverhaltens: dem Angriff auf die Flanken ihrer Beutefische von schräg-hinten. Bislang wurde dieser trophische Polymorphismus als dimorph dargestellt, was bedeutet, dass ausschließlich links- (L-Morphe) oder rechts- (R-Morphe) asymmetrische Tiere vorkommen. Dazwischenliegende Morphologien wurden negiert. Es wird allgemein angenommen, dass L-Morphen darauf spezialisiert sind die rechte Körperseite ihrer Beutefische anzugreifen, während R-Morphen die linke Körperseite bevorzugen attackieren. Bezüglich der Aufrechterhaltung des Polymorphismus wurde die These vorgeschlagen, dass die Beutefische der häufiger angegriffenen Körperflanke mehr Aufmerksamkeit schenken. Dadurch erfährt die häufigere Schuppenfresservariante einen Nachteil zum Nutzen der selteneren Morphe, deren Häufigkeit dann eventuell zunimmt. Bislang glaubte man, dass das Merkmal der Maulasymmetrie alleinig durch die zwei Allele eines einzelnen Gens bestimmt wird (R dominant über L und RR Individuen sind nicht überlebensfähig). Auch wurde das spezielle Verpaarungsmuster, dass ungleiche Tiere sich bevorzugen (L-Morphe mit R-Morphe), als mögliche Erklärung für die Kompensation des RR-Letalitäts-Effektes in
Zusammenfassung
der Populations angeführt. Eine Studie im Rahmen dieser Dissertation sollte Licht in die
dürklich aufkommende Diskussion bringen, die sich auf das Verteilungsmuster der
morphologischen Variation (diskrete oder kontinuierliche Verteilung) und das
Verpaarungsmuster (ungleiche Formen bevorzugen sich oder rein zufällige
Verpaarungen finden statt) dieser Art bezieht. Eine umfangreiche Stichprobe von
annähernd 300 Individuen aus fünf Populationen wurde ihrem natürlichen Lebensraum
entnommen, genau vermessen und in einer Population wurde das Verpaarungsmuster
erneut untersucht. Im Gegensatz zu den weitläufigen Annahmen wird in diesem Versuch
eine kontinuierliche Verteilung der Maulasymmetrie aufgezeigt. Auch ein rein zufälliges
Verpaarungsmuster wird aufgedeckt. Diese Ergebnisse stimmen mit kürzlich
veröffentlichten Studien anderer Forscher überein und stellen die ursprünglichen
Aussagen teilweise in Frage. Die Ergebnisse deuten auf einen erheblichen Anteil von
Umwelteinflüssen auf die morphologische Ausprägung der Asymmetrie dieser Art hin.
Es besteht jedoch die Möglichkeit, dass auch geographische Aspekte eine Rolle spielen
und somit unterschiedliche Muster in Bezug auf die Merkmalsverteilung und das
Verpaarungsmuster in verschiedenen Populationen auftreten könnten. Weitere
Erkenntnisse bezüglich des Verhaltens dieser Art unterstützen die Hypothese der
umweltinduzierten morphologischen Asymmetrie. Unter Zuhilfenahme von adulten
Wildfangtieren und ihren natürlichen Beutefischen wurde im Feldexperiment die
Mehrheit der angegriffenen Körperflanken alleinig durch die ausgeprägte Morphologie
vorhergesagt. Im Zuge eines weiteren Versuchs fand sich bei Jungfischen, die im Labor
aufgezogen wurden, eine sehr starke seitliche Ausrichtung des
Nahrungsaufnahmeverhaltens, insbesondere bei morphologisch nur schwach
asymmetrischen Individuen. Dies deutet darauf hin, dass in dieser Art die
morphologische Asymmetrie durch das Verhalten geleitet wird, was wiederum die
mutmaßliche Rolle von phänotypischer Plastizität in der Ausprägung der
asymmetrischen Maulform unterstreicht, welche kürzlich erst in der aktuellen Debatte
um dieses System herangezogen wurde.

In der vorliegenden Dissertation werden durch die Untersuchung von einigen
innerartlichen trophischen Polymorphismen und eines Farbpolymorphismus bei
Buntbarschen weitere Erkenntnisse bezüglich ökologischer Diversifizierung gewonnen.
Die Dynamik der Aufrechterhaltung derartiger Polymorphismen wird diskutiert und die
etwaige Rolle bezüglich einer möglichen weiteren Aufspaltung erörtert. Dadurch wird
das Verständnis der Entstehung der biologischen Vielfalt ein Stück weit vorangebracht.
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General Introduction

Foreword
Adaptive radiation marks the evolution of ecological and phenotypic diversity in a rapidly speciating lineage (Schluter, 2000; Simpson, 1953). Divergent natural selection arising from differences between environments and competition for limited resources is considered as its main driving force (Schluter, 2000). Species complexes that experience adaptive radiation are believed to be able to explain all diversity on earth (Simpson, 1953). This thesis deals with ecological and evolutionary consequences of trophic- and color polymorphisms in the adaptive radiation of cichlid fishes. Focusing on two charismatic cichlid model systems, one from the Old World and one Neotropical, various ecological and evolutionary aspects of such intraspecific polymorphisms were investigated. The first and major line of enquiry is about the young adaptive radiation of Midas cichlid fishes (*Amphilophus citrinellus* spp.) from Nicaraguan great lakes and crater lakes. In this species complex, questions surrounding the conspicuous gold/dark color polymorphism (Chapters I & II) were investigated and patterns of diversification along the benthic-limnetic axis (Chapter III) were examined. A second line of enquiry focused on the scale-eating cichlid *Perissodus microlepis* from Lake Tanganyika (Africa) and some controversially perceived aspects that back up the understanding of this famous evolutionary model system of a trophic polymorphism were tested (Chapters IV & V).

Although dealing with a single taxonomic group (cichlids), this thesis has implications for a much wider field of study than cichlid research: the study of biodiversity, adaptation, and consequently speciation and adaptive radiation. Hence, the following section is not only aimed to introduce the study systems that are dealt with in this thesis, but also to contextualize them in the larger framework, namely that of speciation and adaptive radiation. After defining of what trophic- and color polymorphisms actually constitute, the question is addressed of how such intraspecific polymorphisms may contribute to (adaptive) speciation processes. Sex-dependent polymorphisms are ignored because in those study systems I have investigated the polymorphic traits occur in both sexes. Whenever it appeared suitable, examples from the wild are given to corroborate the theory.
To keep things focused, I largely restrict myself to examples of freshwater fishes, because -among vertebrates- this group is probably best-studied in that regard and is rich of impressive examples, notably of trophic polymorphisms (Robinson & Wilson, 1994; Robinson & Schluter, 2000; Skúlason & Smith, 1995; Smith & Skúlason, 1996; Wimberger, 1994), but also of color polymorphisms (Gray & McKinnon, 2007; McKinnon & Pierrotti, 2010; Sturmbauer & Meyer, 1992; Wagner et al., 2012).

The larger framework: parallel evolution, speciation & adaptive radiation
The question of how new species arise based on intraspecific diversity has been vigorously debated for a long time and might be the most important question in evolutionary biology (e.g. Coyne & Orr, 2004; Darwin, 1859). Natural model systems such as species complexes have contributed largely to the understanding of how such intraspecific diversity is maintained and how it might translate into speciation (Schluter, 2000; Via, 2009). Monophyletic groups that experience ongoing adaptive radiation (Schluter, 2000) are of particular relevance because the influence of confounding genetic backgrounds and evolutionary histories can be reduced (Schluter, 2001; Via, 2009).

Adaptive radiation is defined as “the evolution of ecological diversity within a rapidly multiplying lineage” (Schluter, 2000), and has occurred in a wide range of taxa and geographical scenarios. A number of key features characterize adaptive radiation. Initially perhaps most important in this regard is the opportunity to explore new habitats and empty ecological niches along with an associated phenotypic response, followed by divergent phenotypic adaptation and finally speciation (Schluter, 2000). For example, Darwin’s finches have diversified on the Galápagos Islands into about a dozen ecologically well-differentiated species (Grant & Grant, 2011), Anolis lizards have produced ecologically-equivalent sets of species in different islands of the Greater Antilles (Losos, 2009), and cichlid fishes have radiated into hundreds of species in each of the East African Great Lakes (Fryer & Iles, 1972; Meyer, 1993b; Ribbink et al., 1983; Sturmbauer, 1998) and to a somewhat smaller, but not less impressive extent in Neotropical water bodies (Barlow, 2002; Kullander, 1998; López-Fernández et al., 2013).
The cichlid fishes are one of the most compelling examples of adaptive radiation because they provide some of the fastest and most impressive patterns of diversification among all currently known adaptive radiations, with more than 2,000 described species (Barlow, 2002; Fryer & Iles, 1972; Turner et al., 2001). Cichlid fishes have been an extensive source of research for the processes of natural selection, sexual selection, and ecological specialization (Meyer, 1993b; Salzburger, 2008). An exceptional diversity with respect to body shape, coloration, foraging adaptations and behavior can be observed (Barlow, 2002; Fryer & Iles, 1972). Cichlids are convincing examples for parallel, convergent evolution, where derived, but ecologically similar phenotypes evolve independently from a common ancestor (Muschick et al., 2012; Stiassny & Meyer, 1999).

Instances of parallel evolution, where similar phenotypes arise independently in different environments from a recent common ancestor, are considered as strong evidence for natural selection in driving diversification and are commonly observed in the framework of adaptive radiation (Elmer & Meyer, 2011; Schluter & Nagel, 1995; Schluter, 2000). Since the nature of parallelism implies several replicates being involved, it is often challenging to deal with different stages of diversification. Natural study settings in this research field are indeed generally comprised by a number of replicates that differ by various ecological and non-ecological factors (Losos, 2009; McKinnon & Rundle, 2002; Østbye et al., 2006). The probably most influential parameters in impacting the diversification process are time allowing for divergence to happen, ecosystem complexity and population-specific characteristics (Gavrilets et al., 2007; Nosil et al., 2009; Orr & Smith, 1998). Arguably, in parallel evolutionary settings with initially similar conditions, e.g. in habitat structure, founder population size and complexity of biotic interactions, predictability should play a certain role (Losos et al., 1998; Mahler et al., in press; Morris, 2006; Morris, 2010; Schluter & McPhail, 1993). This is an important consideration, since it is unlikely that phenotypic convergence and associated reproductive isolation in some cases (Rundle et al., 2000; Servedio et al., 2011) are simply resulting from random processes rather than from natural selection in driving a population apart into the major vacant ecological niches in a particular setting.

Within this framework (of parallel evolution and adaptive radiation), intraspecific polymorphisms, that largely define the scope of this thesis, are often characteristic elements contributing to biological diversity. Such polymorphisms usually
General Introduction

allow for the exploitation of a wider ecological resource spectrum, than in non-polymorphic populations (Smith & Skúlason, 1996). However, the “evolutionary fate” of such intraspecific polymorphisms can be manifold, ranging from a sudden loss to the stable maintenance of the polymorphism over time (Chapters I-V), up to the potential promotion and finally completion of lineage diversification (Chapters I-III).

Polymorphism(s) in the scope of this thesis

Trophic polymorphisms (Chapters III-V) are those that, in the first place, enable or indicate the widening of the resource spectrum explored by a polymorphic population, compared to a non-polymorphic population. In the past, trophic polymorphisms have been largely associated with discrete phenotypes (Skúlason & Smith, 1995; Smith & Skúlason, 1996). However, given that a trophic polymorphism may actually be extremely subtle (Skúlason & Smith, 1995), or only be detected through analyzing a combination of several continuously varying traits (Wimberger, 1994), I extend my definition of trophic polymorphism by adopting the definition of Robinson & Schluter (2000; p. 67), that is based on relative phenotypic variation and that was implemented in the framework of the study of northern freshwater fishes:

“We define a trophic polymorphism as excessive niche-based phenotypic variation within a single population. By excessive, we mean that the variation is greater than seems to be the norm … Variation need not be discrete; it can be continuous as long as it is unusually high.”

Color polymorphisms (Chapters I & II) state the occurrence of two or more genetically determined color morphs within an interbreeding population with the rarest morph being too frequent to result from recurrent mutation (Huxley, 1955). Color polymorphism are generally thought to affect resource use only secondarily, but are primarily impacted by those agents of natural and sexual selection that are related to vision, such as predation and color based assortative mating, respectively.
The origin of polymorphisms

Trophic polymorphisms are usually multivariate traits and are considered to originate from disruptive natural selection on relatively increased phenotypic variance as a result of frequency dependent intraspecific competition for resources between individuals (Robinson & Schluter, 2000; Smith & Skúlason, 1996).

Several processes/circumstances, likely adding up to one another, may trigger the evolution of trophic polymorphisms. The probably most important elements in this regard are intraspecific competition for limited, but underutilized resources and a phenotypic response that relaxes competition by widening the resource spectrum (Skúlason & Smith, 1995; Smith & Skúlason, 1996). Additionally, the presence of heterogeneous and depauperate habitats has been invoked as a favorable circumstance to promote the evolution of trophic polymorphism (Robinson & Schluter, 2000; Smith & Skúlason, 1996). A disruptive selection regime that acts against intermediate phenotypes or that favors rare or extreme phenotypes can cause such an increase in phenotypic variance, up to the evolution of discrete trophic polymorphism (Rueffler et al., 2006; Smith & Skúlason, 1996; Svanbäck & Bolnick, 2007).

It may be important to consider that with increasing similarity of any two given individuals and increasing population densities, also the strength of competition for available and thus limited resources increases (Bolnick, 2004). Similarly, in the literature, trophic polymorphisms are often considered being a result of character release (Robinson & Wilson, 1994; Skúlason & Smith, 1995; Smith & Skúlason, 1996; Chapter III). Further, the establishment of individual specialization (Chapters III-V), an individuals’ restriction of niche exploration relative to the population (Bolnick et al., 2003), may trigger the evolution of trophic polymorphisms because the newly acquired variation in niche use among individuals does not only decrease the relative degree of competition, but is also targeted by natural selection (Bolnick et al., 2003; Rueffler et al., 2006).

When disruptive selection is invoked for the explanation of a phenotypic response and particularly for trophic polymorphisms, it is assumed that the variation in the affected phenotypic traits has a genetic basis. In contrast, some studies have stretched a major and even exclusive environmental contribution in promoting the evolution of trophic polymorphisms (Skúlason & Smith, 1995; Smith & Skúlason, 1996; Wimberger, 1994). In an extreme case, it has been argued, that through a suite of processes, starting with a behavioral plastic response, feeding specialization and
consequently phenotypic divergence may be initiated and strengthened, which in turn may affect mate choice and therefore reproductive isolation (Wimberger, 1994). This line of argumentation bases on the idea of genetic assimilation of phenotypic characters (Waddington, 1953) and is partly addressed in Chapter V. It has been argued that selection of phenotypic variation initially caused by plasticity, may cause a genetic manifestation of phenotypes that were initially only brought about by environmental influences (Baldwin, 1896; Crispo, 2007; Waddington, 1942; Waddington, 1952; Waddington, 1953). Such adaptive phenotypic plasticity is therefore considered as a potential ingredient contributing to phenotypic evolution and of trophic polymorphisms.

The evolutionary origin of color polymorphisms per se, in contrast to trophic polymorphisms, is usually primarily independent of the environment (Huxley, 1955). Therefore, the question possibly merits more attention, how once a color-polymorphic trait arose, it spreads in the population, how the color polymorphism is maintained over time, and whether it has evolutionary consequences. For the sake of interweaving theory with directly relevant examples for this thesis, the polymorphic cichlid study systems investigated here are introduced and their polymorphic nature will be highlighted.

**About the polymorphic Midas cichlid system**

The Neotropical Midas cichlid species flock (*Amphilophus citrinellus spp.*) has become a model system for studying incipient speciation and adaptive radiation (Barluenga & Meyer, 2010; Elmer et al., 2010a; Elmer & Meyer, 2011). In western Nicaragua, several crater lakes have been formed by the accumulation of rain and ground water in volcanic calderas. Although the mode remains speculative (Elmer et al., 2010a; Elmer et al., 2013), in rare and likely independent events, ancestral types of Midas cichlids have managed to colonize at least eight crater lakes from the great lake environment and have further diverged (Barluenga et al., 2006; Barluenga & Meyer, 2010; Elmer et al., 2009; Elmer et al., 2010a; Elmer et al., 2010b; Elmer et al., 2013; Manousaki et al., 2013). Owing to geo-volcanic research, the maximal ages of many crater lakes can be estimated (Kutterolf et al., 2007; Pardo et al., 2008; Siebert & Simkin, 2002). Molecular genetic studies on Midas cichlid population divergence time have proposed colonization scenarios that are plausible with the age estimates from geological studies (Barluenga et al., 2006; Elmer et al., 2013) ranging between ca. only 100 years (Elmer et al., 2010b) and 10,000 years (Barluenga et al., 2006).
These young crater lakes differ from the large Nicaraguan lakes in that they are generally deep, clear, small, isolated (with no in- or outflow), heterogeneous in macro-habitat and species-poor (Elmer et al., 2010a; Waid et al., 1999). Newly formed crater lakes offer unique habitats along with the associated ecological niches to fish that colonize these lakes from surrounding bodies of water. Crater lakes might be considered as “terrestrial equivalent to aquatic islands” (Fryer, 1996; Losos & Ricklefs, 2009) and their importance for speciation research has become more and more relevant in the recent past (Barluenga et al., 2006; Elmer et al., 2009; Elmer et al., 2010b; Gavrilets et al., 2007; Sato et al., 2003; Schliewen et al., 1994; Schliewen et al., 2001). Importantly, in the isolated and depauperate crater lake environment, diversification processes can be studied with less noise compared to other geographical settings. Therefore, each crater lake housing Midas cichlids might be considered as an independent laboratory for evolution with likely similar starting material, i.e. habitat and small founder population size. Further, it was found that diversification can even take place in situ and sympatric speciation has been reported to contribute to the rise of new Midas cichlid species in at least lake Apoyo (Barluenga et al., 2006), and possibly also in lake Xiloá (Elmer et al., 2009). Midas cichlid populations within each crater lake are genetically more similar to each other than to their relatives in any other crater lake (Barluenga & Meyer, 2010).

Also, the Midas cichlid system is rather simple and therefore easier to study compared to other cichlid systems such as the famous radiations in the East African Great Lakes. Throughout, the species complex is phenotypically highly polymorphic. The most striking axes of differentiation relate to body shape differences that gain relevance along the benthic-limnetic axis, to body coloration and to feeding ecology, the latter being manifested in hypertrophied lips and pharyngeal jaw morphology (Figure 1).
A number of polymorphic traits can be observed in the Midas cichlid species complex. A-C depict examples of trophic polymorphisms. A: Body shape along the benthic-limnetic axis. B: Enlarged, hypertrophied lips in contrast to normal lips. C: Lower pharyngeal jaw morphology. D: The conspicuous color polymorphism in overall body coloration.

In lakes Apoyo and Xiloá multiple species can be observed, that reflect diversification along the benthic-limnetic axis (Elmer et al., 2010a; Chapter III). The presence of an open water habitat is of particular relevance for the diversification in the benthic-limnetic context in these crater lakes. The differentiation in the benthic-limnetic axis even under sympatric crater lake conditions is consistent with theory and model predictions (Elmer et al., 2010a; Gavrilets et al., 2007). Benthic species are generally rather shore-associated and have a bulkier body shape than the elongated, arrow-shape like limnetic species that inhabit the open water region (Figure 1A) (Elmer et al., 2010a). Lake Apoyo is the oldest crater lake and houses six described Midas cichlid species, followed by Lake Xiloá which contains four. In crater lakes Apoyo and Xiloá a limnetic species was suggested to originate first from a generalist ancestor, followed by the evolution of multiple benthic species (Kautt et al., 2012). Midas cichlids have already speciated along the benthic-limnetic axis in some crater lakes, but how this
differentiation was achieved remains largely unexplored. Generally, as outlined above, intraspecific competition and individual specialization may favor the evolution of an intraspecific (benthic-limnetic) resource polymorphism through disruptive selection (Bolnick & Fitzpatrick, 2007; Bolnick, 2011; Martin & Pfennig, 2009; Smith & Skúlason, 1996; Svanbäck & Bolnick, 2007; Swanson et al., 2003). It is therefore conceivable that in those lakes (Apoyo and Xiloá), in which completed speciation events can be observed nowadays, speciation was preceded by a trophic polymorphism that was relevant along the benthic-limnetic axis. Focusing on the youngest crater lake replicate, this hypothesis is addressed in Chapter III of this thesis.

In the great lakes of Nicaragua and in crater lakes Apoyeque and Masaya, and possibly Xiloá, Midas cichlids are trophically polymorphic with respect to lip size (Elmer et al., 2010a; Elmer et al., 2010b; Manousaki et al., 2013; McKaye et al., 2002). In those lakes, the normal thin-lipped morph or species is most abundant, but regularly individuals occur that have conspicuously enlarged, hypertrophied lips (Figure 1B). These lips are thought to serve significant functions related to feeding ecology. As a kind of sucking tool, the enlarged lips might help to catch hardly accessible prey items, in particular when feeding from crannies of the rocky shore substrate (Barlow & Munsey, 1976). Others have considered hypertrophied lips in cichlids as probe-like tools, i.e. sensitive structures (Barlow & Munsey, 1976; Fryer & Iles, 1972) or exhibiting an increased olfactory surface (Arnegard et al., 2001; Oliver & Arnegard, 2010), therefore contributing to non-visual prey detection. A recent study has addressed ecological and genetic aspects of parallel evolution of hypertrophied lips throughout the Midas cichlid species complex (Manousaki et al., 2013).

In the Midas cichlid, being trophically polymorphic can also be manifested in the pharyngeal jaw apparatus. Cichlids possess a second set of jaws in the throat allowing them to handle food items that would otherwise be non-exploitable, if the oral jaws were used solely. Their evolutionary success has also been attributed to the versatility in this structure (Fryer & Iles, 1972; Liem, 1973; Meyer, 1990b). The lower pharyngeal jaw (LPJ) has been studied extensively in many species of cichlid fishes and is more or less indicative of the overall pharyngeal jaw apparatus. Conventionally, in Midas cichlids two types of LPJs are distinguished: relatively heavier, rather sturdier LPJs with wider teeth are termed “molariform”, which are in stark contrast to “papilliform” LPJs that are characterized by its rather slender and gracile nature with finer teeth (Meyer, 1990a; Meyer, 1990b) (Figure 1C). Usually, molariform, but not
papilliform lower pharyngeal jaws allow for the cracking of hard snail shells, and papilliform jaws allow to feed more efficiently on soft prey (Meyer, 1989). In lakes with several Midas cichlid species, limnetic species usually have a rather papilliform LPJ morphology, whereas species that are mainly attributed to the benthic habitat exhibit rather molariform LPJs (Barluenga et al., 2006; Meyer, 1990a; Meyer, 1990b; HK personal observation). A plastic component contributing to morphology has been demonstrated (Meyer, 1990a; Muschick et al., 2011). Variability and ecological relevance of Midas cichlid lower pharyngeal jaw morphology is addressed in Chapters I & III of this thesis.

However, apart from those trophic polymorphisms outlined above, the Midas cichlid species complex also exhibits a conspicuous (gold/dark) color polymorphism. Due to this color polymorphism, the Midas cichlid was named after the legend of King Midas, who wished that everything he touched turned into gold. The common dark morph is melanic and cryptic in its natural habitat because of the ability to change its pigmentation pattern, depending on social cues and environment (Barlow, 1976). In contrast, gold individuals which are quite rare (usually less than 10 %) in wild populations have largely lost the black pigmentation during ontogeny and are therefore conspicuously yellowish-orange to reddish colored (Figure 1D). This color polymorphism is likely determined by a single two-allele locus with gold being dominant over dark (Barlow, 1983; Henning et al., 2010). Almost all populations and species are polymorphic in coloration and various ecological and evolutionary relevant color-related aspects are investigated in Chapters I & II.
**About the trophic polymorphism in the scale-eater, *Perissodus microlepis***

Chapters IV & V deal with the scale-eating cichlid *Perissodus microlepis* from Lake Tanganyika. In this species, individuals have remarkably asymmetric mouth orientations that are bent towards the left or the right side to a varying extent (Figure 2).

![Figure 2. The trophic polymorphism in the scale eating cichlid, *Perissodus microlepis*.](image)

The left individual is a L-morph that has the mouth bent to the left, whereas the individual on the right side is a R-morph that has the mouth bent towards the right.

This species feeds predominantly on scales of other fish and attacks its prey fish mostly from behind. It is thought, that the left mouthed morph (or L-morph) preferentially attacks the right flank of their prey fish while the right mouthed morph (or R-morph) attacks the left flank of the prey fish most readily (Hori, 1993; Lee et al., 2012).

However, there are some controversial aspects with respect to the distribution, maintenance, inheritance, and the influence of the environment on mouth asymmetry in this fish (Palmer, 2010; see also Chapters IV & V). Morphological lateralization in this study system has originally been portrayed as a purely genetically determined dimorphism with no intermediate forms. In particular, it was claimed that morphological left-right asymmetry largely follows Mendelian inheritance (R being dominant over L and RR homozygous lethal), and that a disassortative mating pattern would compensate the inheritance pattern to maintain the dimorphism in the population (Hori, 1993; Hori et al., 2007; Stewart & Albertson, 2010; Takahashi & Hori, 2008).
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However, recent quantitative studies suggested a more complex pattern of how morphology is brought about, namely that this trophic polymorphism is likely influenced by major environmental components (Kusche et al., 2012 – Chapter IV of the present thesis; Van Dooren et al., 2010), possibly driven by behavioral laterality (Lee et al., 2012 – Chapter V of the present thesis).

The maintenance of polymorphisms in the population
Polymorphisms are usually thought to be maintained by various types of natural selection, e.g. disruptive and frequency-dependent selection (Skúlason & Smith, 1995; Smith & Skúlason, 1996). Heterosis (heterozygote advantage) has also been invoked in this regard (Fisher, 1922; Gray & McKinnon, 2007). Additionally, some authors have stretched the putative role of phenotypic plasticity (Pfennig et al., 2010; West-Eberhard, 1986; West-Eberhard, 1989; Wimberger, 1994) in bringing about a stable maintenance of a polymorphism over time. Arguably various processes are likely being involved in many systems, but the relative contributions of those remain usually largely unexplored (e.g. Gray & McKinnon, 2007 and Chapters I & II of this thesis).

Heterosis refers to the situation where intermediate or heterozygous individuals exhibit a higher fitness than extreme or homozygous ones. Probably the most famous example for heterotic effects is a case of human disease biology: sickle cell anemia (Allison, 1954). Individuals carrying a genetic variant of α-hemoglobin regularly suffer from anemia caused by a (sickle-like) deformation of the red blood cells and associated effects such as the block of capillaries. Individuals which are homozygous for the sickle cell hemoglobin have severely decreased survival rates. However, it has been demonstrated, that despite this severe disadvantage for homozygous individuals bearing the sickle cell hemoglobin, heterozygous individuals are more resistant to malaria than either homozygous type. This relative fitness advantage of heterozygous individuals has been invoked to maintain the polymorphism in populations that are exposed to malaria (Allison, 1954).

Interestingly, unpublished data suggest (Henning, 2012) that maintenance of the Midas cichlid color polymorphism may in addition to others processes (Chapters I & II), also exhibit a heterotic component. For example, Henning showed that genetically heterozygote individuals tend to enter the transition phase later in ontogeny, i.e. at larger sizes, than homozygous gold individuals. Therefore it is conceivable that heterozygous individuals might gain a relative survival advantage, compared to homozygous gold
individuals due to reduced predation pressure imposed by gape-limited predators (Chapter II).

In those cases where the maintenance of a polymorphism over time is mainly achieved by disruptive selection, it is usually assumed that the polymorphism is of ecological relevance to the exploration of its respective resource spectrum. Such ecological relevance can be reflected for instance in a mismatch of extreme phenotypes or either morph in the respectively different environment or in selection against maladaptive intermediate phenotypes or hybrids in either parental environment (Nosil, 2012). This implies that a given phenotype or morph is relatively most fit in its native habitat. One major aspect from the stickleback literature deals with the repeated evolution of sympatric benthic and limnetic species pairs. Within this framework plentiful evidence is lend for the role of ecological relevance of morphology achieved through divergent natural selection, and it has been suggested that this is crucial for the maintenance of those species pairs, e.g. (McPhail, 1994; Schluter, 1995). In Chapter III, differentiation along the benthic-limnetic axis is investigated in the Midas cichlid system and aspects relating to the ecological relevance of morphology and signatures of disruptive selection are tested. Another study on the conspicuous color polymorphism in the Midas cichlid system has revealed associated ecological consequences that are likely genetically determined in parts (Chapter I). This suggests that Midas cichlid color morphs explore different ecological niches, somewhat enabling their stable co-existence over time and indirectly indicating that divergent selection might contribute to the reported differentiation between color morphs.

When frequency dependent selection is invoked to explain the maintenance of a polymorphism over time it is assumed that the fitness of a given phenotype depends on its relative frequency in the population, compared to alternative phenotypes. A widely perceived example is the scale-eating cichlid *Perissodus microlepis* from Lake Tanganyika and its lateralized foraging behavior (Chapters IV & V). In this system individuals are ecologically highly specialized in that they have asymmetric jaw orientations and mouth morphologies that enable them to snatch of scales from their preferred prey fish flanks most efficiently (Figure 2). The right-mouthed morph preferentially and more successfully feeds from the prey fish left flank and the left-mouthed morph from the prey fish right flank. Negative frequency dependent selection has been invoked to explain the stable morph ratio in the population over time around equal abundance (Hori, 1993). The rationale was that at a point in time the common
morph suffers a fitness disadvantage, because the prey fish are more alert against the most frequently attacked body flank (Hori, 1993), therefore the rare morph is favored and hence increases in frequency. Another putative example of the role of frequency dependent selection in maintaining the color polymorphism in Midas cichlids imposed by predators is addressed in the discussion of Chapter II.

The putative role of polymorphisms in lineage diversification

In various study systems (Jonsson & Jonsson, 2001; Liem & Kaufman, 1984; McKay, 1980; Meyer, 1990a; Meyer, 1993a; Robinson & Wilson, 1994; Robinson & Schluter, 2000), and theoretical models (Felsenstein, 1981; Forsman et al., 2008; Gray & McKinnon, 2007; Rosenzweig, 1978; Smith, 1966; West-Eberhard, 1986; West-Eberhard, 1989) trophic- and color polymorphisms have been invoked to effectively promote reproductive isolation and hence speciation, even under sympatric conditions.

Generally, a variety of processes potentially leading to the restriction of gene flow between morphs can cause reproductive isolation, and hence speciation (Coyne & Orr, 2004; Futuyma, 2009; Nosil, 2012). It is assumed that if sympatric speciation would be achieved based on a polymorphism, speciation is usually preceded by a phase of stable maintenance over time, e.g. (Smith, 1966). In this light, distinguishing between the scenarios of whether a polymorphism is “simply” purely maintained over time or actually constitutes a transitional stage towards diversification seems somewhat difficult. However, it was considered that only under very particular conditions a polymorphism may lead to ecological speciation in sympatry, namely that a genetic mechanism is present that links the response under divergent selection to reproductive isolation (Nosil, 2012; Smith, 1966). Such genetic correlation can in principle be achieved by “magic traits” (Gavrilets, 2004), where the trait under selection pleiotropically affects reproductive isolation or by genetic linkage, where the genes that code for the traits under selection and those that are involved in mate choice are physically linked and therefore inherited together (Nosil, 2012; Servedio et al., 2011; Chapter I). Discriminating between magic traits and linkage is impossible unless the genetic architecture of the trait of interest is clarified in detail. Nevertheless, in a recent review paper (Servedio et al., 2011) it has been pointed out that strong physical linkage can mimic magic traits in their effects. Further, in that study (Servedio et al., 2011) “automatic magic traits” and “true magic traits” have been distinguished.
“Automatic magic traits” encompass those scenarios where different phenotypes/morphs are favored in different habitats and mating is habitat specific (habitat isolation), or where temporal isolation is achieved through the coupling of reproductive isolation to temporal differences in spawning time, that e.g. might have been induced by specialization on different food types, which itself have different peak abundances throughout the season. A number of putative examples have been listed of putative “true magic traits”, where mating is based on the trait under divergent selection itself (Servedio et al., 2011).

Throughout the literature, genetic correlation between the traits under divergent selection and those that affect mate choice has been suggested to serve as a powerful mechanism contributing to the evolution of reproductive isolation between morphs, and consequently of speciation, also in sympatry (Bolnick & Fitzpatrick, 2007; Gray & McKinnon, 2007; McKinnon & Pierrotti, 2010; Nosil, 2012; Servedio et al., 2011). For instance body size in sticklebacks (Conte & Schluter, 2013) and body shape in *Gambusia* fishes (Langerhans, 2007) are known to be under divergent selection pressure and mating largely bases upon body size or shape in those systems. In the example dealing with the color polymorphism in Midas cichlids, assortative mating between morphs of the same coloration can be observed in the wild (Elmer et al., 2009; McKay & Barlow, 1976). In Chapters I & II it is suggested that the Midas cichlid color polymorphism is correlated with ecological differences between morphs that might be partly genetically determined. The suggested genetic correlation of ecologically-relevant traits and mate choice bears great potential in mediating sympatric divergence in this system.

Phenotypic plasticity has also been invoked to contribute to the maintenance and evolution of divergent phenotypes in a population, and finally to speciation (Pfennig et al., 2010; West-Eberhard, 1986; West-Eberhard, 1989; Wimberger, 1994). Such plasticity increases the available phenotypic variation of a given population and has also been demonstrated in cichlid trophic structures (Greenwood, 1965; Gunter et al., in press; Meyer, 1987; Meyer, 1990a; Meyer, 1990b; Muschick et al., 2011; Wimberger, 1991; Wimberger, 1992). For example, it was argued that plasticity could enable a (polymorphic) population to endure ecological crunch times (Meyer, 1987; Meyer, 1990a; Meyer, 1990b), i.e. periods with limited food supply or temporal unavailability of certain types of resources requiring the exploration of alternative food types. Besides, its potential contribution to reproductive isolation was acknowledged if plasticity
affected mate choice relevant traits (Greenwood, 1974; Meyer, 1990a; Meyer, 1990b; Wimberger, 1991; Wimberger, 1992; Wimberger, 1994). However, it appears likely that in many systems multiple agents that act in concert, such as selection and plasticity, contribute to the effective maintenance of polymorphisms in the wild (Figure 3).

Figure 3. A generalized framework about the origin, maintenance and evolutionary consequences of trophic- and color polymorphisms. The graph was drawn following (Smith & Skúlason, 1996) and reflects the major points raised throughout the introduction. Reference is given to the investigated cichlid systems and individual chapters in this thesis. Given the complexity of the topic, the scheme should be seen as an approximation somewhat representing the “consensus” of several other investigators and the author.
Expected contributions of this thesis to the broader field of research

Adaptive radiation is a stunning phenomenon that encompasses speciation bursts, following ecological opportunity (Schluter, 2000). How such diversification processes are initiated at the population level and what role polymorphisms play in there are questions still not fully resolved in biology. Various freshwater fishes, including cichlids have been studied in that regard. Cichlids comprise more than 2,000 described species (Turner et al., 2001). Their stunning diversity in various aspects such as ecology, eco-morphology, coloration and behavioral traits make them an ideal study system to infer those processes and elements that have contributed to the unparalleled evolutionary success of this vertebrate family, and consequently those that lead to diversification. It is therefore assumed, that the knowledge gathered from cichlid fishes to infer evolutionary processes, is of general interest and transferable to other systems.

One major gap of knowledge refers to the maintenance and the impact of discrete and sex-independent color polymorphism on lineage diversification (Maan & Sefc, 2013; McKinnon & Pierrotti, 2010; but see Wagner et al., 2012). Focusing on the Midas cichlid system, Chapters I & II address the long standing question of how such a color polymorphism may be maintained at low frequencies over time and investigate the possibility that the color polymorphism might contribute to the evolution of reproductive isolation in sympatry. Given the mode of inheritance (Henning et al., 2010), the mating pattern (Elmer et al., 2009; McKay & Barlow, 1976) and the reported intraspecific advantages of being gold (Barlow, 1973; Barlow, 1983), it is conceivable that being gold may also come at a disadvantage in other disciplines that might contribute to the maintenance of gold at low frequency across populations. Furthermore, theory suggests (Kirkpatrick & Ravigne, 2002) that gold and dark must exhibit ecological consequences in order that divergence in sympatry can be achieved, as has been suggested (Elmer et al., 2009; McKay & Barlow, 1976; McKay, 1980).

In Chapter I a complex ecological component to the Midas cichlid color polymorphism is revealed across multiple populations. Eco-morphological differentiation is likely partly genetically correlated to coloration. In the light of the high degree of color assortative mating in the system (Elmer et al., 2009), this genetic correlation of ecologically relevant traits and coloration might potentially facilitate sympatric differentiation, and possibly speciation. In the scope of adaptive radiation research, this is an unparalleled empirical finding.
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Chapter II shows color-differential predation by a major natural predator of Midas cichlids. The potential role in maintaining the color polymorphism at relatively low frequencies is discussed.

In Chapter III a different aspect of Midas cichlid diversification is investigated. Throughout freshwater fishes, differentiation along the benthic-limnetic axis is a commonly observed phenomenon and a striking example of parallel, convergent evolution (Robinson & Wilson, 1994; Robinson & Schluter, 2000). Chapter III focuses on the putative differentiation of Midas cichlids along the benthic-limnetic axis in the youngest Nicaraguan crater lake, Asososca Managua. This study, that combines ecological and genetic approaches, was initiated because previous studies, e.g. (Elmer et al., 2010a) suggested that in this lake (sympatric) diversification processes along the benthic-limnetic axis might be occurring, as has been documented for the populations of the older crater lake Apoyo and Xiloá (Barluenga et al., 2006; Elmer et al., 2010a). Such putative early stages of divergence form the most appropriate context to study the evolution of trophic polymorphisms and therefore for the understanding of ongoing diversification processes. In this young polymorphic crater lake population, the ecological significance of morphology along the benthic-limnetic axis is demonstrated in terms of individual specialization and morphology-diet correlations. Given the data, the evolution of a benthic-limnetic resource polymorphism is discussed in the light of character release, followed by speculations about the evolutionary consequences of this polymorphic system.

Chapters IV & V focus on aspects relating to discreteness and evolutionary maintenance of the lateralized trophic polymorphism of *Perissodus microlepis*, the scale-eating cichlid from Lake Tanganyika. Traditionally, this species has been considered as a clear-cut case of a genetically determined trophic dimorphism (Hori, 1993; Hori et al., 2007; Stewart & Albertson, 2010) and a specific (disassortative) mating pattern has been invoked to back up the inheritance pattern (Hori, 1993; Hori et al., 2007; Takahashi & Hori, 2008). However, recent work pointed at inconsistencies across studies, e.g. (Palmer, 2010; Van Dooren et al., 2010), and the possibility of environmental influences on morphological laterality have been invoked, e.g. (Van Dooren et al., 2010), therefore also questioning the assumed genetic model. In Chapter IV several geographically distinct populations of this species were sampled to verify the match with one of the key assumption in this system, namely that negative frequency-dependent selection maintains the polymorphism at about equal abundance over time.
Also morphological asymmetry was quantitatively assessed to distinguish the presence of a discrete dimorphism from that of a rather continuous variation (Van Dooren et al., 2010). Using a larger sample size than in previous studies (Takahashi & Hori, 2008), the mating pattern was reinvestigated. Altogether, morphological asymmetry was found being continuously distributed and the mating pattern was random. This is in line with recent claims (Van Dooren et al., 2010), that the system is probably not exclusively determined by a single major locus and that environmental influences might contribute to morphological laterality in this species. In Chapter V, the lateralized feeding behavior was investigated and found to be strong, even in laboratory-raised juveniles that are poorly asymmetric with respect to morphology. It is suggested that behavioral lateralization might induce or contribute to morphological manifestation of asymmetry in this system and not the other way around as has been initially claimed (Hori, 1993).
Chapter I

A conspicuous color polymorphism facilitates diversification in a young adaptive radiation

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To be submitted to Science
Abstract

Color polymorphisms are common in nature. However, in the framework of adaptive radiation their significance in promoting ecological diversification remains largely unexplored. The rapidly speciating Midas cichlid species complex consists of multiple independent populations that have a striking (gold vs. dark) color polymorphism in common and mating is color assortative. We test the potential impact of this color polymorphism on intraspecific diversification in this young adaptive radiation. Using a unique combination of morphological and ecological data from multiple replicated lakes in the wild, we found that this color polymorphism strongly correlates with ecological differentiation in the wild. *gold* Midas cichlids are higher-bodied, have more robust lower pharyngeal jaws, and occupy a more benthic ecological niche compared to sympatric, conspecific *dark* individuals. A common garden experiment in the laboratory indicated partial genetic correlation of the color polymorphism and eco-morphology in the same pattern as was found in the wild populations. Given that the color polymorphism functions as a trait, both being subject to assortative mating and having ecological consequences, sympatric speciation might be facilitated. The herein revealed role of this color polymorphism is an innovative finding for adaptive radiation research and might refine adaptive radiation theory in general.

Introduction

John Maynard Smith argued that under very particular ecological and genetic conditions a stable intraspecific polymorphism could result in sympatric speciation via niche differentiation and reproductive isolation (Smith, 1966). Almost five decades later, numerous theoretical insights into the mechanisms that permit sympatric speciation have been accumulated. However, empirical evidence for it remains scant because of the challenge in identifying the polymorphism, the role of disruptive selection, evidence for sympathy, and its true rarity in nature (Bolnick & Fitzpatrick, 2007). One of nature's most conspicuous, and sometimes dramatic, intraspecific polymorphisms refers to coloration. Color polymorphisms, or the occurrence of two or more genetically determined color morphs within an interbreeding population (Huxley, 1955), are relatively widespread throughout the animal kingdom. However, the significance of color polymorphisms in promoting diversification is controversial and has been rarely inferred, although some studies have made some progress in this regard (Gray &
Color polymorphisms potentially cause great impact on various intra- and interspecific processes such as communication and color assortative mating, the vulnerability to predation (Hubbard et al., 2010) beside exhibiting further ecological consequences (Forsman et al., 2008; McKinnon & Pierrotti, 2010).

One particularly powerful mechanism through which rapid sympatric speciation may be facilitated is genetic correlation of the traits that are under divergent selection and those that affect mate choice and therefore reproductive isolation (McKinnon & Pierrotti, 2010; Nosil, 2012; Servedio et al., 2011). Such genetic correlation either means that the genes that affect sexually- and ecologically relevant traits are physically linked and therefore inherited together or that the trait underlying mate choice pleiotropically affects ecology (“magic trait” (Gavrilets, 2004)). For example, assortative mating by phenotype matching for body size has been demonstrated in various stickleback species pairs (Conte & Schluter, 2013; McKinnon et al., 2004; Nagel & Schluter, 1998), Gambusia mosquito fishes for body shape and other examples summarized in a recent review (Servedio et al., 2011). Interestingly, in some studies such genetic correlation has also been reported for color polymorphic systems such as has been reported for mimetic wing color patterns in Heliconius butterflies (Jiggins et al., 2001; McKinnon & Pierrotti, 2010).

However, the understanding of the evolutionary processes associated with color polymorphisms is often somewhat impaired in many systems due to the sometimes complex or poorly understood underlying genetic architecture of color polymorphic traits, and sex chromosome linkage of mating preferences and the color polymorphism itself (McKinnon & Pierrotti, 2010). Thus, an ideal study system to address the ecological and evolutionary consequences of color polymorphisms would incorporate multiple replicates, sex-independence, and a simple genetic basis of a complex but easily determinable color-polymorphic phenotype associated with assortative mating. Here we examine such a system, while revealing its relevance for the study of ecological and evolutionary divergence associated to color polymorphisms.
In the young adaptive radiation of Midas cichlid fishes (*Amphilophus citrinellus-labiatus* species complex), we assessed the ecological consequences and the potential of a conspicuous binary (*gold*/dark) color polymorphism in shaping parallel sympatric diversification. We focused on ten color polymorphic populations from two Nicaraguan great lakes and four crater lakes (Figure 4).

**Figure 4. Investigated Midas cichlid color morphs and their geographic distribution.**
In Nicaragua, a number of small crater lakes have been colonized by Midas cichlids from the great lakes of Nicaragua (Lake Managua and Lake Nicaragua). Color-polymorphic populations of Midas cichlids can be found in most crater lakes as well as in the ancestral great lake population at low frequencies. Ten color-polymorphic populations were analyzed in this study.
Because the crater lakes were colonized from the ancestral great lake environment in only the last 100 to 6 kya (Elmer et al., 2010a; Elmer et al., 2010b; Elmer et al., 2013), and the Midas cichlids in each crater lake are genetically more similar to each other than to those of any other population (Barluenga & Meyer, 2010), the populations can be seen as “natural experiments” in evolution each of them being a replicate with a potentially different evolutionary outcome. Midas cichlids are polymorphic not only in coloration but also in trophic niche: driven by disruptive natural selection on body shape that allowed them to exploit different niches they have adaptively radiated into species or trophic morphs in sympatry (reviewed in (Elmer et al., 2010a)), sensu other freshwater fishes such as postglacial sticklebacks, whitefish, and arctic char (Mousseau et al., 2000; Robinson & Wilson, 1994; Schluter, 2000). Midas cichlid fishes are so named because of the conspicuous, discrete and sex-independent color polymorphism in which one morph is amelanic and therefore has completely yellowish to orange body and fin color ("gold") while most fishes are melanic, being dark-gray with vertical bars and dark fins ("dark") (Figure 4). This color polymorphism is a Mendelian trait and determined by a single two-allele locus with gold dominant over dark and almost complete penetrance (Barlow, 1983; Henning et al., 2010). All individuals are initially indistinguishable and start life with the typical dark-greyish coloration that is mainly based on melanophores and which can be modified according to the individual’s motivational status and the background (Barlow, 1976). Before or around the age of sexual maturity (ca. 0.5 to 3 yrs or ~10 cm length) gold individuals lose their dark coloration because the melanophores in the skin die off and thereby their entire body coloration becomes orange-gold (Dickman et al., 1988; Henning et al., 2013). The gold phenotype has been shown to be correlated with social behavior, with golds exhibiting a domination advantage that translates into an increased growth rate when competing with dark morphs (Barlow, 1973; Barlow, 1983). Other behavioral traits have been suggested to be linked to gold, such as color assortative mating in the wild (Elmer et al., 2009; McKay & Barlow, 1976) and breeding in slightly deeper waters than dark morphs (McKay & Barlow, 1976; McKay, 1980). A recent analysis of two ecologically differentiated but sympatric sister species in crater lake Xiloá found that, despite breeding in sympatry, at same depths and season, pairs were significantly color assortative (95% of A. sagittae pairs and 77% of A. xiloaensis pairs) (Elmer et al., 2009). A striking implication of this color-based sexual isolation was incipient speciation; there was significant population genetic divergence in sympatry (Fst = 0.03,
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$p < 0.001$; individuals could be assigned to color >80% successfully based on multilocus microsatellite allele frequencies alone (Elmer et al., 2009)). In contrast, earlier data on Midas cichlids in the great lakes of Nicaragua found only weak population genetic divergence between sympatric color morphs (Barluenga & Meyer, 2004).

The sympatric, conspecific color polymorphism of Midas cichlids occurring across multiple populations that vary significantly in body shape (Elmer et al., 2010a) has been a long-standing dilemma. From a behavioral ecology perspective it is unclear, how gold morphs persist at low frequency within species given that they have a competitive advantage over dark morphs (Barlow, 1973). From an evolutionary biology perspective, the maintenance of a stable polymorphism, such as this color polymorphism, requires a complex target of selection or else, by stochastic effects, the rare morph will be lost (Smith, 1962). Because gold and dark are genetically determined traits and there are mixed matings, sexual selection theory argues that divergence based on the color polymorphism alone is not possible, unless the color polymorphism has ecological consequences (Kirkpatrick & Ravigné, 2002). Here we show for the first time that there is a complex, multivariate ecological component to the stable color polymorphism of Midas cichlids and that this is evidenced in the wild and in laboratory experiments.

Specifically, we tested whether ecological divergence is associated with the color polymorphism in Midas cichlids, that might contribute to the maintenance of the color polymorphism and to the reported genetic differentiation (Barluenga & Meyer, 2004; Elmer et al., 2009). Given the prevalence of the color polymorphism occurring in multiple similar but independent crater lakes, we expected parallel and similar directions of ecological diversification between color morph pairs across these evolutionary replicates. To test for genetic correlation of the color polymorphism and ecologically-relevant traits that would significantly facilitate sympatric speciation (McKinnon & Pierrotti, 2010; Nosil, 2012; Servedio et al., 2011), we conducted a common-garden approach under controlled laboratory conditions. Throughout our study, we found consistent ecological differentiation between color morphs in the wild. Our laboratory experiment suggested partial genetic correlation of the color polymorphism and eco-morphological traits.
Material and Methods

Specimen collection

Individuals were sampled from ten different populations of Midas cichlids from the great lakes and crater lakes of Nicaragua by gill-netting or harpooning between 2001 and 2012. In the field, standardized photographs of the left body side of each fish were taken from directly above. Tissue samples (fin, muscle) were collected and stored in pure ethanol. All specimens were taken as vouchers (head or whole body) and stored in 70 per cent ethanol.

Figure 5. Definition of eco-morphological measurements for the gold/dark project.
Left side: Anatomical description of body landmarks: LM 1 = tip of snout; LM 2 = most posterior point of the lips; LM 3, 4 = most anterior and posterior margin of the eye; LM 5 = intersection of operculum and ventral body outline; LM 6 = anterior base of dorsal fin; LM 7, 8 = dorsal and ventral base of pectoral fin; LM 9 = anterior base of pelvic fin; LM 10, 11 = anterior and posterior base of anal fin; LM 12 = posterior base of dorsal fin; LM 13, 14 = dorsal and ventral base of caudal fin; LM 15 = midpoint of the caudal fin origin; LM 16 = nostril; LM 17 = dorsal end of pre-operculum; LM 18 = dorso-caudal origin of the operculum. Right side: Anatomical description of LPJ landmarks and caliper measurements: LM 1,2 & LM 10,11= tips of posterior and lateral processes of upper horn; LM 3,9 = points of highest curvature in upper horn base; LM 4,8 = points of closest intersection between horn base and dentition area; LM 5, 7 = points of highest curvature near the mid-point of the two adjacent landmarks; LM 6 = posterior-most point of lower pharyngeal jaw suture; LM 12-15 & 18-21 = points of highest curvature near the mid-point of the two adjacent landmarks; LM 16,17 = anterior tips of lower horn processes; LM 22, 23 = most posterior teeth of the “external line” of the dentition area; LM 24 = point where the suture meets the dentition area. Caliper measurements LPJ width, length and depths are also illustrated (grey arrows). Jaw and body shape outlines modified from Manousaki et al., 2013.
Ecological assessment of color morph differentiation

a) Body Height Index and Body shape

Eighteen landmarks (LM) describing body shape of 1,354 individuals were digitized from standardized photographs in tpsDig v. 2.16 (Rohlf, 2010a) by a single investigator (HK; Figure 5, modified from (Manousaki et al., 2013)). Body Height Index (BHI) is the relative fraction of body height (Figure 5: distance LM 6 to LM 9) divided by standard length. BHI was calculated from inter-landmark distances that were obtained in PAST v. 2.16 (Hammer et al., 2001) for each individual. BHI is a condensed proxy of overall body shape and was therefore used in the global logistic regression (below).

Multivariate shape analyses on body shape of 1,177 individuals (Table S1) were performed in MorphoJ v. 1.05c (Klingenberg, 2011), largely following a previous study on Midas cichlid body shape differentiation (Elmer et al., 2010a). Body shape data exhibited significant allometric effects (5.04 % of shape explained by centroid size; \( p < 0.0001 \)), thus the allometry-corrected shape data (regression residuals) were used in downstream analyses. Discriminant function analysis (DFA) (Albrecht, 1980; Mardia et al., 1979; Timm, 2002; Zelditch et al., 2012) was used to test for significant differentiation between mean color morph body shapes within each population.

b) Lower pharyngeal jaw shape analyses

Standardized photographs from dorsal view were taken from extracted and cleaned lower pharyngeal jaws \( (n = 465; \) Table S2) from directly above using a digital camera. 24 homologous landmarks, consisting of 12 fixed and 12 semi-landmarks were defined that describe external LPJ shape along with the dentigerous area (Figure 5). Digitization was done tpsDig v. 2.16 (Rohlf, 2010a) by a single investigator (HK) from the specimens photographs. Semi-landmarks were slid in tpsRelw v. 1.49 (Rohlf, 2010b) in orthogonal projection mode with 10 iterations. Slid semi-landmarks were treated as true homologous landmarks in MorphoJ v. 1.05c (Klingenberg, 2011). Object symmetry was taken into account and the symmetric component of shape variation only was considered as our trait of interest (Klingenberg et al., 2002). A correction for allometric effects on LPJ shape was performed by regressing Procrustes coordinates on centroid size (4.99 % explained; \( p < 0.0001 \)). Regression residuals were used in downstream analyses that were conducted analogous to body shape analyses. In a complementary approach that covers traits that were not assessed by the geometric morphometric approach, external characteristic LPJ features of 456 specimens (lateral horn width, jaw
length, keel depth; Figure 5) were measured using a digital caliper and jaws were weighted. Cube root normalization was applied to LPJ weight. LPJ-variables were standardized and corrected for allometric effects. Linear discriminant analysis was performed in R v. 2.11.1 (R Core Team, 2012) to test for differences in LPJ traits between each pair of color morphs. LPJ weight in these 456 specimens was representative of overall LPJ morphology and was therefore used in the global logistic regression analysis (below).

c) Stable isotope analysis
Stable carbon (δ¹³C) and nitrogen (δ¹⁵N) isotope ratios were used to infer trophic divergence between color morphs. Generally, δ¹³C indicates the relative source of benthic and limnetic carbon and thus macro-habitat, whereas δ¹⁵N corresponds to the relative trophic level (Post, 2002). A small piece of muscle tissue was extracted from dorsal musculature of 298 ethanol preserved specimens (Table S3) and dried for ca. 48 hours at 55°C. Samples were ground in individual sealed tubes and a 1.0 - 1.5 mg subsample was weighted. Analyses were done by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) at the Limnological Institute (University of Konstanz). δ¹³C-values were corrected for lipid content. Two sample t-tests were consulted to test for mean isotopic differentiation between color morphs within each population of Midas cichlids.

Comparative data analysis of BHI, LPJ weight and stable isotopes
a) Binary logistic regression on eco-morphology
Pair-wise comparisons suggested a major and population independent axis of differentiation between color morphs. To test for a consistent and species complex-wide eco-morphological differentiation between color morphs, logistic regression with a binominal error distribution was used and conducted in R v. 2.15.1 (R Core Team, 2012). Being gold (“1”) or not (“0”≡ dark) was set as response variable and allometry corrected BHI or LPJ weight was set as explanatory variable. To test for an overall effect, each population was modeled as random factor. The glmer-function (package “lme4” (Bates, 2005)) was used to fit the models. The sim-function (package “arm” (Gelman & Hill, 2007)) was used for simulation of 5,000 values from the posterior distribution of the model parameters. Inference was drawn, based on the 95 % Credible Interval (CrI), which is the Bayesian analogous to confidence interval.
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Conventionally, if zero is not included in the Bayesian 95 % CrI, an effect is considered as “clear” (Amrhein et al., 2012).

b) Linear Mixed Effects model on stable isotope signatures

Paired t-tests suggested a systematic difference in the trophic level $\delta^{15}$N between color morphs (Figure S1). Therefore, a linear mixed effects model was consulted. The modeling approach was selected to test for a global species-complex-wide difference in the trophic level $\delta^{15}$N between color morph pairs, while accounting for $\delta^{13}$C. Therefore, in the model “$\delta^{15}$N” was treated as response variable and “color” and “$\delta^{13}$C” where defined as fixed effects, i.e. as explanatory variables, while each population was treated as random factor. Residual analysis was done visually to confirm that the model assumptions were adequately met (Figure S2). The lmer-function (package “lme4”(Bates, 2005)) was used to fit the models. The sim-function (package “arm”(Gelman & Hill, 2007)) was used for simulation of 5,000 values from the posterior distribution of the model parameters. Inference was drawn, based on the 95 % Credible Interval (CrI) as described above.

Test for genetic correlation of the color polymorphism and eco-morphology

In this common garden approach 107 siblings of an F2-intercross (heterozygous for the gold-locus) between gold/dark F1-Hybrids were raised together in the same tank throughout their life in the animal research facility of the University of Konstanz and later in a heated 10 000 l outdoor tank to a standard length of ca. 11 cm. This size range is similar to that of wild-caught adult fish in our field collection (ca. 14 cm). All fish were processed the same day and eco-morphological color morph differentiation has been assessed as described above for the field specimens. Phenotypically 68 individuals were gold, eight of which were in transition phase and 39 individuals were scored as dark individuals. Two microsatellites that are in strong linkage disequilibrium with the gold locus (Henning et al., unpublished data) were amplified using established conditions. This was done to discriminate in the phenotypically dark cohort between those individuals that were truly genetically dark and those that were untransformed gold individuals. In theory, the gold to dark offspring ratio of a mating involving two heterozygous gold parents should be close to 3:1. Correspondingly, we found 79 genetically gold and 28 genetically dark morphs in our sample. Most genetically gold individuals were already transformed or still in transition. However, 11 genetically gold
individuals could only be identified by the microsatellites.

Results and Discussion

Body shape differentiation between color morphs

To test if sympatric, conspecific color morphs, of different Midas cichlid populations differ in body shape, we analyzed 1,177 wild-caught individuals. Our sampling encompassed most crater lakes and great lakes that were known to harbor color polymorphic populations (Elmer et al., 2010a) or of which we provide first evidence here along with sufficient sample sizes (Figure 4; Table S1, Figure S1). Using geometric morphometric analysis of body shape based on 18 descriptive landmarks, we found that across the ten populations combined, color morphs differed significantly from each other in body shape (gold and dark pooled; Procrustes distance = 0.01, Hotel-T2 = 248.06, p < 0.0001), with the gold morph being higher-bodied and having a larger head relative to the dark morph (Figure 6). When we examined population-by-population, this trend was evident across nine of the ten populations, though in three populations sample sizes were small (n ≤ 7) because of the extreme natural rarity of either the gold or the dark morph in some populations (Figure S1, Table S1). Altogether, this reveals that color morphs throughout the Midas cichlid complex differ consistently in body shape.

Figure 6. Body shape differentiation between Midas cichlid color morphs.

The left panel depicts wireframe outline graphs to illustrate the shape changes between the group means. On average, gold morphs were higher bodied and had a larger head than dark morphs (scale factor = 10). Binary logistic regression revealed a consistent eco-morphological differentiation throughout the ten sampled study populations using Body Height Index (BHI) as a proxy for overall body shape. The histogram (middle panel) indicates absolute values in the range of the linear predictor, whereas the density curve (right panel) represent relative values, and is therefore corrected for unequal sample sizes of gold and dark morphs.
To test for body shape differentiation between color morphs independent of population-level effects on shape, we used allometry corrected Body Height Index (BHI), which is a robust univariate proxy of overall body shape. We found a clear effect for gold being higher bodied than dark morphs, irrespective of population (mean of posterior distribution = 15.77 (95 % Credible Interval = 9.66 to 21.93)). We interpret the logistic regression coefficients following Gelman and Hills “divide by 4 rule” (Gelman & Hill, 2007). Practically, when considering a shift in BHI of ca. 0.1 units in our sample, which is about half the range of BHI (-0.1 to 0.09, spanning 0.19 units) the probability of being gold increased by maximal 39.4 %. In other words the probability of being gold and not dark for a given individual as predicted by the model clearly increased with higher BHI values (Figure 6). Overall, this confirms our finding from the pair-wise comparisons; throughout the species complex gold morphs differ in body shape from sympatric, conspecific dark morphs in that golds are generally higher-bodied.

Trophic ecology differs between color morphs

Across freshwater fishes in general (Clabaut et al., 2007; Robinson & Wilson, 1994; Robinson & Schluter, 2000; Schluter, 2000) including Midas cichlids (Elmer et al., 2010a), differentiation in body shape is associated with the exploration of different ecological niches, with more high-bodied fishes being associated with a more benthic niche. To test whether the observed body shape differentiation between gold and dark morphs is associated with ecological differentiation, we tested individual-level characteristics of trophic ecology: lower pharyngeal jaw shape, size and weight, and stable isotopic signatures of δ13C and δ15N.

Lower pharyngeal jaw (LPJ) size and shape is strongly associated with diet in cichlids (Kusche et al., 2013; Muschick et al., 2012). In Midas cichlids, stronger, broader and heavier jaws with wider teeth are more associated with a mollusk-rich diet that requires snail crushing (molariform jaws), while more gracile LPJ with finer teeth and less dense bone are found in fishes that eat a more piscivorous or planktivorous diet (papilliform jaws) (Meyer, 1990a; Meyer, 1990b) and are associated a more limnetic niche, e.g. (Barluenga et al., 2006). To quantify the LPJ of Midas cichlids across the ten sampled color polymorphic populations, we conducted a multivariate analysis of the LPJ shape (geometric morphometrics of 24 homologous landmarks in 465 individuals; Figure 5, Table S2). In a complementary approach that was in accordance with the multivariate approach, a linear discriminant analysis was conducted on caliper
measurements of LPJ width, length, keel depth, and weight on the same set of specimens (Figure 5). Consistent with the body shape differentiation, we found that color morphs throughout the pooled ten populations significantly differed in LPJ shape (gold and dark pooled; Procrustes distance = 0.01, Hotel-T^2= 67.73, p < 0.0001; Figure 7), with gold morphs tending to have more molariform and heavier LPJs compared to dark morphs (Figure 7; Figure S1). To test for the complex-wide LPJ differentiation between color morphs irrespective of the population, we used logistic regression with a binary error distribution (gold/dark) and considered LPJ weight (corrected for allometry) as a univariate proxy of LPJ morphology (e.g. Kusche et al., 2013 - Chapter III of the present thesis). We found a clear effect for gold morphs having heavier jaws than dark morphs (mean of posterior distribution = 1.50 (95 % Credible Interval = 1.05 to 1.97)). Hence, following (Gelman & Hill, 2007), a relative shift of LPJ weight of 1 unit within the range of LPJ weight in our data (-1.93 and 2.23, spanning 4.16 units) corresponded to an increase in probability of being gold by maximal 37.5 %. This indicates that gold morphs had consistently heavier and therefore rather molariform lower pharyngeal jaws than dark morphs and that the probability of being gold increased with the relative weight of the jaw (Figure 7).

Figure 7. Differentiation in trophic ecology between Midas cichlid color morphs. Lower pharyngeal jaw (LPJ) shape differentiation between Midas cichlid color morphs is depicted in the left panel (scale factor =10), indicating a rather robust (molariform) LPJ morphology for gold morphs compared to dark morphs. A consistent differentiation in LPJ weight across the study populations was revealed using binary logistic regression. LPJ weight was used as a proxy of overall jaw morphology. The histogram (middle panel) indicates absolute values in the range of the linear predictor, whereas the density curve (right panel) represent relative values, and are therefore corrected for unequal sample sizes of gold and dark morphs.
This also suggests that gold Midas cichlid morphs might be able to process a more benthic, mollusk-rich diet relative to conspecific, sympatric dark morphs, a finding which awaits further confirmation by analysis of devoured prey items. So far, in a single investigated pair of color morphs (Amphilophus xiloaensis) a slightly higher proportion of mollusk remains was observed in stomachs of gold morphs (Elmer et al., unpublished data). However, our field samples were collected during the breeding season, where the fish become territorial and feeding is largely restricted and additionally records of instantaneous feeding may be not representative of long-term diet. Because of these issues and in the light of our sporadic sampling of prey we focused on long-term inference of diet.

To test whether the eco-morphological differentiation found between color morphs corresponded to a systematic trophic divergence in diet, we analyzed stable isotope signatures of carbon and nitrogen integrated in muscle tissue of 298 individuals across all populations (Table S3). Isotopic ratios reveal long-term integrated signatures of diet, therefore indicating ecological niche, with $\delta^{13}C$ representing the relative source of benthic and limnetic carbon and thus macro-habitat, whereas $\delta^{15}N$ corresponds to the relative trophic level (Post, 2002). Most pairs of sympatric color morphs consistently differed significantly in the trophic level $\delta^{15}N$, but not in $\delta^{13}C$ (Figure S1). Therefore, to test for any systematic differentiation in the trophic level across the ten investigated populations, we tested whether $\delta^{15}N$ differed between color morph pairs, while accounting for $\delta^{13}C$ and considering each population as a random factor. We found that consistently and independently of population, gold morphs fed at a lower trophic level than dark morphs (model coefficient: -0.63 ‰ $\delta^{15}N$ (95 % Credible Interval = -0.86 ‰ $\delta^{15}N$ to 0.39 ‰ $\delta^{15}N$) (Figure 8). This suggests that throughout the species complex, sympatric gold and dark color morphs exploit different trophic levels and therefore different ecological niches.
Figure 8. Stable isotope differentiation between Midas cichlid color morphs.
The fitted regression lines of the linear mixed effects model are indicated by solid lines for both color morph groups. Dotted lines indicate the 95% credible interval obtained from the posterior model distribution. gold morphs consistently fed at a relatively lower trophic level ($\delta^{15}N$) than dark morphs, independent of the population and while accounting for $\delta^{13}C$. Symbols indicate the groups as listed in the legend.

Test for genetic correlation of the color polymorphism and eco-morphology

Our analysis of wild fishes sampled from ten populations of Midas cichlids found strong sympatric, conspecific differences between color morphs in body shape and other indicators of trophic niche. However, these observed differences might be primarily either genetically or environmentally induced, or both. If eco-morphological differences are environmentally induced, for example because of differential resource use associated with the social dominance of golds when interacting with dark (Barlow, 1973; Barlow, 1983), then we would not expect a difference between color morphs to be carried over in a common garden experiment where food is identical for all individuals. If the eco-morphological trait difference between color morphs in the field is genetically correlated with being gold, there should be a detectable difference between color morphs despite common garden conditions.

To assess whether the eco-morphological variation we found in the wild is genetically correlated to coloration, we conducted a common garden experiment using captive fishes ($n = 107$ siblings) derived from an F2-intercross (heterozygous for the gold-locus) between gold/dark F1-Hybrids (Henning et al., 2010). At the time of analysis (when fishes were ~11 cm standard length; similar to our field collections), 79
individuals were genetically gold (though 11 golds were still dark in body coloration) and 28 individuals were genetically and phenotypically dark. We analyzed each individual for body shape and LPJ shape, using the same methodology that was previously applied to the wild-caught specimens.

Common garden experiment

![Body shape and LPJ shape charts](image)

Figure 9. Outcome of the common garden experiment of Midas cichlid color morphs.

This approach was conducted to reveal any genetically determined eco-morphological differentiation between color morphs. Albeit sibling fish of both color morphs have been raised in the same environment, there is still significant body and LPJ shape differentiation between them. The color-morph differentiation in these traits among sibling fish is similar to that observed in the wild (Figure 6, Figure 7 & Figure S1). As in the wild, the gold morph has a larger head and a rather robust LPJ morphology compared to the dark morph.

The common garden experiment strongly suggested at least a partial genetic correlation of the color polymorphism and the eco-morphologically relevant traits body shape and LPJ shape. The eco-morphological differentiation between laboratory raised gold and dark sibling fish was similar though slightly lower than that in most wild populations (body shape: Procrustes distance 0.010, Hotellings $T^2 = 92.58$, $p = 0.006$, jaw shape: Procrustes distance 0.014, $T^2 = 59.36$, $p = 0.007$; Figure 9). As in most wild-caught populations, gold morphs had a relatively larger head and sturdier pharyngeal jaws than dark morphs (Figure 9).
The persistent eco-morphological differentiation in body- and LPJ shape of laboratory-raised gold and dark sibling fish suggests that the color polymorphism has potentially direct ecological consequences (Meyer, 1990a) and that eco-morphological differentiation in body- and LPJ shape is not entirely environmentally induced. The suggested genetic correlation, be it mediated by linkage disequilibrium or through pleiotropic effects, is likely to play a major role in the maintenance of the color polymorphism and incipient diversification in this system (Barluenga & Meyer, 2004; Elmer et al., 2009). Mechanistically, genetic correlation would allow for intraspecific divergence based on the color polymorphism while promoting reduced environmental competition between both morphs, through the agents of natural selection. If the color polymorphism would not be associated with ecological consequences, then divergence based on color assortative mating alone is very unlikely since one morph is likely to outcompete the other, leading to the loss of the color polymorphism within only few generations (Kirkpatrick & Ravigné, 2002; Kondrashov & Shpak, 1998; Kondrashov et al., 1998).

**Differences in crater vs. great lakes**

The populations we investigated in this study consist of gold/dark color morph pairs inhabiting substantially different environments, with the deep, clear-watered, and young crater lake environment strongly differs from that of the turbid and shallow waters of the Nicaraguan great lakes and the crater lakes themselves differing in size, age, and depth (Elmer et al., 2010a). Given this striking differences in macro-habitat, it is very likely that natural and sexual selection pressures affecting Midas cichlid body coloration are different across environments. In both environments, diet differentiation between color morphs was consistently present. However, in the clear and deep crater lakes, in particular in lake Xiloá, color morphs exhibited higher degrees of eco-morphological differentiation than is present in the great lake environment. This is consistent with population genetic analyses, that found significant differentiation between sympatric color morphs in a crater lake and only little in the great lakes (Barluenga & Meyer, 2004; Elmer et al., 2009).
Among the ten investigated populations, the average body- and also LPJ shape differentiation between color morphs was higher in crater lake populations than in great lake populations (crater lakes: mean Procrustes distances $^{\text{Body shape}} = 0.019$, range = 0.011 - 0.025; mean Procrustes distances $^{\text{LPJ shape}} = 0.02$, range = 0.007 - 0.036 $n = 6$; great lakes: mean Procrustes distances $^{\text{Body shape}} = 0.016$, range = 0.010 - 0.030; mean Procrustes distances $^{\text{LPJ shape}} = 0.017$, range = 0.006 - 0.037, $n = 4$; Figure S1). This raises the interesting possibility that divergent selection forces, assuming they were responsible in bringing about the herein presented ecological differentiation between color morphs, might be stronger in the crater lake environment rather than in the turbid and shallow great lake environment and that the extent of assortative mating and the divergence in different ecological niches may be different across environments. Possibly, the great lake environment does not promote ecological diversification of color morphs as much as the crater lake environment does. This hypothesis is also supported by the species richness within crater lakes (e.g. up to six sympatric, endemic species) relative to the great lakes (Elmer et al., 2010a).

Conclusions
In our study of multiple independent evolutionary replicates, we found considerable degrees of intraspecific ecological differentiation between sympatric color morphs. The idea that a stable polymorphism is maintained at low frequency implies the exploitation of different fitness peaks in the adaptive landscape and is fully consistent with adaptive radiation theory (Gavrilets, 2004; Martin & Wainwright, 2013; Schluter, 2000) and our data. The often clear ecological differentiation between Midas cichlid color morphs can eventually bear witness that shifts towards previously unoccupied fitness peaks have occurred, through which stable coexistence of both color morphs might be achieved, also providing the raw material for ongoing diversification processes (Gavrilets, 2004; Gray & McKinnon, 2007; Schluter, 2000). The revealed genetic correlation between the color polymorphism and ecologically-relevant traits bears great potential to facilitate sympatric ecological speciation in this young adaptive radiation. Through the integrative combination of data both based on extensive field collections using independent natural replicates and collected under controlled laboratory conditions, this study provides a strong case, for the ecological and evolutionary consequences of color polymorphism in the wild.
Acknowledgements

We thank all the helpers for their efforts in the field, in particular R. Rayo and H. Recknagel. S. Stockmaier kindly assisted in the lab. Thanks to J. Sieling and M. Wolf for technical support. We thank F. Henning for providing unpublished genetic markers to identify untransformed gold individuals and comments on this manuscript. We thank the Stable Isotope Group of the Limnological Institute at the University of Konstanz. We are grateful to F. Korner-Nievergelt for her advice with statistical analysis. Special thanks to the participants of the 2012 IMPRS writing course held at the SCENE field station in Loch Lomond (Scotland) for comments on previous versions of this manuscript. Funding was provided through various grants of the Deutsche Forschungsgemeinschaft to AM, an Alexander von Humboldt and NSERC fellowship to KRE, a European Research Council (ERC) advanced grant “GenAdap” to AM and of Deutsche Cichliden Gesellschaft to HK.
Chapter II

Color-differential predation by a visually hunting predator and its potential implications for a young adaptive radiation of crater lake cichlids

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To be submitted to Biological Journal of the Linnean Society
Abstract
Predation is a major agent of natural selection. From a visually-hunting predator’s perspective conspicuous prey items can be most likely easier spotted that inconspicuous ones. However, few studies have demonstrated differential predation related to prey conspicuity. Throughout the color-polymorphic (gold/dark) Midas cichlid species complex (Amphilophus citrinellus spec.), gold morphs occur at relatively low frequencies (< 10 %), although at the intraspecific level gold is believed to exhibit a competitive advantage compared to dark and gold being genetically dominant over dark. In a controlled laboratory experiment, we tested whether a major natural predator of Midas cichlid color morphs differentially predates on color variants of goldfish (Carassius auratus auratus), which were used in analogy to Midas cichlids due to their similarity. We simultaneously displayed size-matched pairs of prey fish to the predator, each consisting of one conspicuous (gold, transformed) and one relatively camouflaged and inconspicuous (grey, untransformed) individual and the time until both prey fish were attacked was registered. We found a clearly increased risk for the gold morph being attacked first (ca. 70 %). Once a predator decided to attack one of the two prey fish the time to attack was found being indiscriminant for both color variants. Our results suggest two things that are not mutually exclusive: First, assuming the predator immediately attacks upon prey detection it must have perceived the gold morph first. Second, the predator exhibited a preference or higher motivation to attack the gold, and therefore the more conspicuous prey fish. Relevantly, the increased risk of the gold, transformed morphs being attacked first in our experiment suggests that being gold in the wild has a pronounced natural selection cost for Midas cichlids. This might then explain why gold is low frequency in the wild, although it is genetically dominant over dark and given the proposed competitive advantage of gold compared to dark.

Introduction
Predation can be considered a major driving force of evolutionary change because it is a complex and ubiquitous agent of natural selection that shapes and regulates all aspects of population structure. This is because predation causes differential survival and therefore reproductive success of individuals within a given population, potentially altering the populations’ characteristics over time (Darwin, 1859; Endler, 1986; Mayr, 1963; Schluter, 2000). Through the effects of predation a variety of evolutionary
processes can be induced (Langerhans, 2007), ranging from morphological evolution (Brönmark & Miner, 1992; Reimchen, 1994), the modification of sexually relevant signals (Stoddard, 1999), to the maintenance of color polymorphism (Bond, 2007) and its potential involvement in mediating speciation (Gray & McKinnon, 2007; Hugall & Stuart-Fox, 2012). A population is considered being color polymorphic when it is comprised of two or more genetically determined color morphs (Huxley, 1955). Although relatively common, the impact of color polymorphisms on diversification remains at most controversial (Gray & McKinnon, 2007; Hugall & Stuart-Fox, 2012).

It may be perceived as “common sense” that visual predation affects conspicuously colored prey items of a given kind more heavily than cryptic ones. This is because conspicuous coloration may be costly resulting in a decreased individual survival probability due to predation, as has been demonstrated by numerous studies, e.g. (Endler, 1978; Endler, 1980; Godin & McDonough, 2003; Kettlewell, 1973; Vignieri et al., 2010). Similarly, efficient crypsis is an essential mechanism of predator avoidance and consequently of survival and largely depends on the predators capacity to visually perceive their prey (Crawley, 2009; Endler, 1978; Endler, 1980). An individual can be considered as cryptic “if it resembles a random sample of the background perceived by predators…” (Endler, 1978).

The study of color-polymorphic populations/species forms an appropriate context to study the evolutionary consequences of visual predation on population structure, since it allows to study those processes with less noise compared to studies above the species level (Godin & McDonough, 2003; Kettlewell, 1973; Vignieri et al., 2010). The complex population dynamics of color polymorphic populations, e.g. often driven by frequency-dependent processes (Allen & Greenwood, 1988; Endler & Greenwood, 1988), might lead to substantially different evolutionary outcomes ranging from stable maintenance of the color polymorphism, to fixation of one variety or complete loss (Forsman et al., 2008; Gray & McKinnon, 2007).

This study seeks to address the evolutionary consequences of differential visual predation on a young color-polymorphic adaptive radiation of cichlid fishes from the Neotropical crater lake Midas cichlid species complex (Amphilophus citrinellus spec.) from Nicaragua. Cichlid fishes constitute one of the most species rich families of vertebrates with ca. 2,000 – 3,000 species and form a model system for the study of speciation and adaptive radiation. Cichlids are famous for their exuberant morphological diversity and spectacular coloration, including color polymorphism
Despite of a rising interest in clarifying the role of coloration-related divergence, the effects of predation on color-polymorphic cichlid species remain surprisingly scarcely inferred (Maan & Sefc, 2013); but see (Annett, 1989; Maan et al., 2008). The Midas cichlid is usually cryptically-greyish colored (dark) with spotted and barred patterns. However, across various species in the complex there is a conspicuous and low frequency orange-golden colored morph (gold; Figure 1, Figure 4, Figure 10). It is a long-standing question, why natural populations only have few gold individuals and why the gold morph does not go extinct or become more common due to natural selection. The color polymorphism is determined by two alleles at a single locus and its segregation largely follows Mendelian inheritance with the gold-determining Allele being dominant (Henning et al., 2010). During ontogeny, at approximately 10 cm of length, gold individuals undergo a transition phase where they sequentially lose their melanophores and therefore black pigmentation, until orange and red pigmentation prevail permanently (Barlow, 1976; Dickman et al., 1988; Henning et al., 2010). Competition is very strong in the lakes and only very few and successful breeding pairs will raise their brood to an independent stage (McKay & Barlow, 1976; McKay, 1977). The ichthyofauna in the Nicaraguan crater lakes is relatively depauperate (Elmer et al., 2010a; Waid et al., 1999) and substantially dominated by Midas cichlids (Barlow, 1976). However, in most crater lakes housing Midas cichlids, various piscivorous predator species called Jaguar cichlids co-occur (notably Parachromis managuensis), that constitute the top of the suite of natural predators of Midas cichlid color morphs in the wild (Barlow, 1976). The presence of the color polymorphism throughout multiple populations at low frequencies, its conspicuity and the fact that gold and dark are genetically determined traits suggests that there might be natural selection consequences of being gold. We are here addressing a predation-related aspect of this hypothesis. Specifically, we are here testing whether differential visual predation through Parachromis managuensis might contribute both to the maintenance of the conspicuous (gold/dark) color polymorphism, and to the rarity of the gold morph throughout the Midas cichlid species complex. This was done under the rationale that effective crypsis of the common dark Midas cichlid morph in the crater lake environment is achieved through two major contributing features, which in the gold morph are absent. The first component contributing to crypsis is countershading, which in fish (Denton, 1971), including Midas cichlids (Barlow, 1976), is associated with relatively darker dorsal and paler ventral coloration, effectively blurring the body outline of a given individual.
against predators view form below and also above, therefore increasing crypsis. The second is background matching, which in Midas cichlids is achieved through the physiological ability to regulate various pigmentation patterns depending on mood and the environment (Barlow, 1976). In the gold Midas cichlid morph, those elements are absent, therefore increasing conspicuity and likely causing increased levels of predator attention.

Using color variants of goldfish (*Carassius auratus auratus*) as alternative prey fish to Midas cichlids (Figure 10) we inferred from a controlled laboratory-based approach, whether *Parachromis managuensis*, differentially predates on prey fish that differ only in conspicuity due to coloration. We project our findings onto the situation in the wild and discuss how this may influence the maintenance of the Midas cichlid color polymorphism in the wild at low frequencies.

**Figure 10. Color variants of the Midas cichlid and the common goldfish.**
Top: The common melanic (*dark*) Midas cichlid color morph is depicted on the left side and the right side shows an individual of the rare *gold* morph. At the initial stage, *dark* and *gold* individuals are until orange pigmentation prevails. Midas cichlid photographs are a courtesy of Frederico Henning. Bottom: two representative goldfishes of the laboratory strain used in this study are shown. The left photograph depicts a still untransformed individual, whereas on the right an individual after completion of the color transition into the universally known form is shown. In this study of color-differential predation by a predatory cichlid, goldfish were used as analogous prey to Midas cichlids.
Chapter II

Material and Methods

Experimental Design

Twelve predators (*Parachromis managuensis*) were used in this experiment. Six specimens were descendants of wild-caught fish, originating from El Salvador, that were kept in captivity for multiple generations by Peter Buchhauser. Six more specimens were wild-caught as larvae in February 2012 in crater lake Asososca Managua in Nicaragua, where *Parachromis managuensis* co-occurs with Midas cichlid color morphs of both types (personal observation).

All predators were raised in the animal research facility of the University of Konstanz. Experiments were approved by the German Federal Animal Welfare Board (permission number 35-9185.81/G-11/12) and conducted between May 2012 and May 2013 in the animal research facility of the University of Konstanz. Predators were isolated and acclimatized for several weeks in independent and standardized fish tanks (> 200 l) under artificial light regime (12h*day⁻¹). Each tank had the bottom covered with gravel and was equipped with two halves of a clay pot and a piece of wood that served as shelter, apart from an aquarium heater and a filter. All experimental tanks that were used in this study were regularly maintained by professional animal caretakers. Maintenance operations included the weekly replacement of ca 50 % of the water the cleaning of the filter. Throughout the experiment, the color and the clarity of the water were consistently similar to that of clear Nicaraguan crater lakes that hold color morphs in the wild (crater lakes Apoyeque, Masaya, As. Managua, As. Léon, Xiloá; personal observation during dry seasons 2010-2013).

Predators were raised on a steady flake food base. The predator’s diet was regularly upgraded by living fish. This was done to enhance the growth rate of the smallest predators and to make sure they would readily ambush and hunt fish upon participation in the experiments. Living prey fish were randomly selected depending on availability and comprised a variety of species, colors and pigmentation patterns. The prey fish included Midas cichlid juveniles (*Amphilophus citrinellus* spec.; grey, vertical bars/spots), Zebrafish (*Danio rerio*, silverfish blue-green, horizontal stripes) as well as various species of Poeciliids (*Xiphophorus* spec. & *Poecilia* spec., whose color ranged from uniformly grey to uniformly red with some intermediate stages and various color patterns). Experiments were started when predators readily fed on live fish of ca. 3 cm total length. The average predator measured around 20 cm when the experiment started (the mean total length measured after the experiment series was 202 mm, ranging from
Due to the temporal and quantitative unavailability of small and size-matching Midas cichlid color morphs, a laboratory strain of the common goldfish (\textit{Carassius auratus auratus}) was used in the experiment in analogy to the Midas cichlid color morphs. Similar to the Midas cichlid, \textit{Carassius auratus auratus} also changes coloration during ontogeny from an inconspicuous greyish-greenish form into the well-known and conspicuous gold-orange form. The overall coloration of both ontogenetic stages of goldfish, before and after color transition, is similar as in Midas cichlids (Figure 10, Figure 11).

This experiment has been designed under the assumption that these ontogenetic changes in body ground coloration cause a difference in perceptibility by a natural aquatic predator of Midas cichlids and that the orange morph in either species, is more conspicuous to this predator. Prey fish pairs were compiled in a way such that contrasting body ground coloration prevailed within each pair. Therefore, each pair consisted of one untransformed individual (grey, relatively camouflaged) and one already transformed individual (gold, relatively conspicuous) of goldfish. Prey fish pairs were size-matched as close as possible by size and weight to exclude the potential impact of prey size on the predator’s foraging decisions.

In order to back up the choice of using goldfish in our experiment in analogy to Midas cichlids, the spectral reflectance patterns of Midas cichlid color morphs and those of goldfish were evaluated. Measurements were done using a spectrometer (Ocean Optics QE65000 Scientific-grade Spectrometer). Using standardized conditions, three individuals each of either type of Midas cichlid color morph were measured and two individuals each of transformed and untransformed goldfish. For each individual, a small dorsal flank area was measured three times and the data obtained from these replicates were averaged out in two levels, first by individual and later at the level of color morph/species.
Figure 11. Reflectance of Midas cichlid and goldfish color morphs.
Depicted are normalized reflectance spectra of Midas cichlids and goldfish color variants, uncorrected (A) and corrected (B) for the conditions, under which the experiment series has been conducted (T8-light). Although in (A) all four groups have their reflectance peaks around 610 nm, only the orange morphs of either species show a
well-defined peak in this orange part of the light spectrum, highlighting their prevailing conspicuous coloration. In contrast, the melanic morphs of either species rather approach a plateau indicating that they reflect light of throughout the range of the considered wavelengths ($\lambda = 500 \text{ nm} - 650 \text{ nm}$) rather uniformly and therefore should appear rather greyish to an observer. In addition, the untransformed goldfish has a steep increase in reflectance already around 550 nm, therefore rendering it rather greenish-greyish. In (B), the T8-light-corrected data are shown. T8-light has two characteristic peaks in the greenish and the orange part of the light spectrum (at $\lambda$ of ca. 545 nm and ca. 614 nm). The relative differences in reflectance between those peaks are indicated by peak height. Similar to the finding in (A) the melanic morphs of either species exhibit only very subtle differences in reflectance intensity, seen under T8-light. Orange morphs of either species exhibited a clearly increased level of relative reflectance at the second characteristic T8-light peak ($\lambda$ ca. 614 nm) compared to the first green one ($\lambda$ ca. 545 nm, indicated by arrows). The difference in reflectance intensity between the T8-peaks were smaller in the transformed goldfish than in the Midas cichlid gold morph, making the transformed goldfish somewhat “less intense gold” than the Midas cichlid. In the figure legend photographs of typical gold and dark Midas cichlid morphs and the goldfish color variants are communicated for visualization purposes. The Midas cichlid pictures are a courtesy of Frederico Henning.

Predators were individually tested with a simultaneously displayed pair of gold and grey prey fish and the time to attack was measured for both prey fish and censored after 5 min. The first cohort of six predators was tested 20 times each in 2012, but one Predator (Predator #6, ID = 276093400100854) died after Trial 8 due to an accident. In 2013, the second predator cohort consisting of six wild-caught specimens was tested 12 times each due to limitations in goldfish supply and given the clear response obtained from the first predator cohort. As a general procedure the experimental trials were conducted by two experimenters. In each trial one experimenter attracted the predator towards one corner of the fish tank by simulating typical hand signs involved in regular feeding procedures, whereas the other experimenter (HK) simultaneously released both prey fish from his hand, at a point in time when the predator appeared particularly involved in the diversionary tactic. From the release of the prey fish on, the diversionary tactic was instantly aborted, and the time to attack of both prey fish was measured using a stop watch to the nearest second.

Occasionally, predators where tested more than once per day (twice: $n = 39$; three times: $n = 9$, five times: $n = 1$) with a prey fish pair, providing a relatively high level of remaining appetent behavior after the preceding experimental trials, as judged by the experimenters and depending on the availability of small goldfish. Our sample of 180 trials excludes 21 conducted experimental trials that did not met the experimental requirements and therefore were not considered being valid by the two experimenters.
These trials involved those, where the predators sight to both prey fish was restricted \((n = 8)\) because the predator’s focus was still directed towards the area, where the diversionary tactic took place, while one or both prey fish hide behind fish tank equipment such as the filter, the heating or the clay pot or Aquarium wood that served as shelter for the predator. In other trials that were also discarded, one or both prey fish showed random escape behavior upon release into the water and therefore both prey fish did not stay closely together, such that the predator could perceive both simultaneously or was likely to be exceedingly attracted by this panic-like escape behavior \((n = 8)\). In three further discarded trials the observers could not clearly discriminate which prey fish has been attacked first because both fish were devoured almost at the same time. Two remaining trials were erroneously conducted and later discarded because the planned sample size per predator was already achieved and therefore omitted from the analysis. Altogether, in 12 of those discarded 21 trials, the untransformed, grey goldfish would have been eaten first, 8 trials were in favor of the transformed individual and one remained truly indiscriminant.

Data analysis

The data were analyzed in two complementary ways that require different methodology and that allow focusing on different aspects concerning the predators foraging behavior, i.e. (i) “choice by prey fish color” as well as (ii) ”time to attack depending on prey fish color, irrespective of the attack order”.

In the first line of enquiry (i), binary logistic regression with a binominal error distribution was used to model the predation event per se \(\text{response variable: gold prey fish has been attacked first (“1”) or not (“0”)}\) under the null hypothesis that both prey fish categories exhibited equal chance (50 %) of being attacked first. We used “Predator-ID” as a random factor to account for repeated measurements and to test for an overall effect, i.e. independent of the predator. The \textit{glmer}-function (package “\textit{lme4}” (Bates, 2005)) was used to fit the models. The \textit{sim}-function (package “\textit{arm}” (Gelman & Hill, 2007)) was used for simulation of 5,000 values from the posterior distribution of the model parameters. Inference was drawn, based on the 95 % Credible Interval (CrI), which is the Bayesian analogous to confidence interval. Conventionally, if zero is not included in the Bayesian 95 % CrI, an effect is considered as “clear” (Amrhein et al., 2012). We also tested for the influence of the prey fish weight ratio as a fixed effect, but following Bayesian inference we not find any effect of weight on the attack probability.
(estimate: 0.93; 95 % CrI = -0.87 to  2.74), therefore prey fish weight was not included in the model. This result is also in support of our experimental design that was addressed to exclude the effect of prey fish weight as much as possible.

In the second line of enquiry (ii) we were interested in whether the time to attack depended on prey fish color, irrespective of the order of which prey fish has been eaten first. A linear mixed effects model has been used to address this question. The nature of censored survival data usually causes an increasing variance with increasing time (Cox & Oakes, 1984) and our experiment does conform to the pattern. To stabilize the variance throughout the time series, a Box-Cox power transformation was applied to the measured attack time using the boxcox-function (package “MASS” (Venables & Ripley, 2002)). In our model the transformed time until the predator attacked the prey fish was modeled as response variable and the color and the attack-order of the prey fish was set as fixed effect along with their interaction. Again, aiming at testing for a generalized response pattern, Predator-ID was included as a random effect in the model. The residual analysis revealed that the model assumptions of this model were met adequately (Figure S3). Bayesian inference was drawn as described above.

In a complementary approach, a survival analysis was conducted in terms of a time-to-event exponential model (cox-proportional hazard model). This type of “survival analysis” was run using the R-functions coxme (package “coxme” (Therneau, 2009)) and Surv (package “survival” (Therneau & Lumley, 2009)). With help of the coxph- and the survfit-function (package “survival” (Therneau & Lumley, 2009)), the model fit was visually confirmed by assessing the Kaplan-Meier Survivorship curves (Efron, 1988; Kaplan & Meier, 1958) obtained from the previously fitted model to the fitted regression lines of the four groups (gold\textsuperscript{1st}, grey\textsuperscript{1st}, gold\textsuperscript{2nd}, grey\textsuperscript{2nd} (Figure S4). The Kaplan-Meier Survivorship curve depicts the estimated proportions of survivorship likelihood of over time. For this additional analysis, inference (frequentist) was drawn from the model summary.
Chapter II

Results

Throughout the experiment the number of gold morphs ($n = 126$) being eaten first exceeded that of grey morphs ($n = 54$) (Table 1). A predator-independent color-based differential attack probability was revealed. Given a goldfish prey individual was of the conspicuous gold and not of the grey type it exhibited an increased hazard of being eaten first by the predator, which clearly deviated from 50 %. From the binary logistic regression analysis the probability of being attacked first when gold was estimated as 70.05 % (95 % CrI = 63.13 % to 76.35 %).

The time until an attack was effected, was indiscriminant for both prey fish categories, when “attack order” was corrected for (model fit of the linear mixed effects model = 0.31; range 95 % CrI = -1.39 to 2.03). Albeit, untransformed morphs were slightly earlier caught by the predator than gold morphs within each category of capture order given the model fit and 95 % CrI in parentheses: gold$^{1st}$ = 8.45 (6.79 to 10.14), grey$^{1st}$ = 8.15 (6.17 to 10.11), gold$^{2nd}$ = 19.76 (17.75 to 21.79) and grey$^{2nd}$ = 19.42 (17.75 to 21.07) (Figure 12).

Table 1. Overview about the outcome of the conducted predation experiment.

Twelve individuals of the predatory cichlid Parachromis managuensis were tested in standardized 1:1 assays of transformed (rather conspicuous, gold) and non-transformed (rather cryptic, grey) goldfish (Figure 10). Differences in sample size were due to fluctuating prey fish availability and due to predator mortality. The first cohort of six specimens was tested 20 times each, except Predator #6 (276093400100854), which died after the eighth trial. The second cohort consisted of six more specimens and was tested twelve times each.

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Figure 12. Time to attack, corrected for attack order.
The means (dot) along with 95% Credible Interval (bars) of the posterior model distributions are depicted for the estimated time to attack within each category of attack order ("first, second") of both prey fish color morphs ("gold/grey"). When attack order was corrected for, the time to attack for both color morphs was indiscriminant.

All twelve predators showed the same attack pattern as has been inferred from the model, but differences in their attack latency (Figure S5). The complementary survival analysis confirmed our previous finding obtained from the linear mixed effects model, that the time until an attack time was conducted, was not different for both prey fish categories (p-value = 0.64), when “capture order” was corrected for.

The analysis of spectral reflectance analysis, also under experimental (artificial T8-light-conditions), revealed general accordance of the reflectance properties of color morphs of either species, therefore suggesting a general match of their color properties from an observers (predators) point of view (Figure 11). Orange morphs of either species had a clear and pronounced reflectance peak around a wavelength of ca. 610 nm (orange), whereas in the melanic morphs of both species all wavelength were rather reflected simultaneously (shades of grey). However, the untransformed goldfish
additionally exhibited a relative increase in reflectance in the greenish section of the light spectrum ($\lambda$ ca. 550 nm). A similar pattern was observed, when focusing on the spectral reflectance curves that were corrected for artificial light conditions, similar to that under which the experiment has been conducted. Further, the relative difference in reflectance between the relevant orange T8-peak ($\lambda$ ca. 614 nm) and the greenish one ($\lambda$ ca. 545 nm) is bigger in the gold morph of the Midas cichlid than in the transformed goldfish. This suggests, that transformed goldfish appear “less orange” than Midas cichlid gold morphs, indicating that the result obtained may be considered being conservative.

**Discussion**

The experiment presented here has been conducted to evaluate the hypothesis that *Parachromis managuensis*, a major predator of *gold* and *dark* Midas cichlids in the wild, displays differential predation against prey fish. In our laboratory set-up we tested pairs of color-dimorphic goldfish in analogy to Midas cichlids.

The transformed goldfish within each pair of prey, exhibited a clearly increased probability of being attacked first by the predator, i.e. 70.05 % (95 % CrI = 63.13 % to 76.35 %). Within each category of “capture order, the time to attack was indiscriminantal for both prey fish categories, when “capture order” was corrected for (mean = 0.31; range 95 % CrI = -1.39 to 2.03. This means that once the predator initiated an attack towards one of the prey fish, both categories of prey fish color were equally fast attacked. The initial attack bouts were consistently more likely directed towards the gold and therefore relatively more conspicuous prey fish. Assuming, a transferability of this experiment to field conditions and to the Midas cichlid system, this strongly suggests, that our data either reflected an enhanced perceptibility of gold morphs compared to dark morphs by the predator. Alternatively, under the assumption that both prey fish were simultaneously perceived by the predator, a clear preference towards attacking gold morphs might be existent in *Parachromis managuensis* or both. This also suggests that the gold morph, when simultaneously exhibiting the risk of predation with a dark morph, exhibits a selection disadvantage in the sense of natural selection pressure by co-occurring predators.

We assume transferability of our findings to wild conditions. In this regard, an investigation of our predator’s visual capabilities is warranted along with the comparison of spectral reflectance signals emitted by Midas cichlid color morphs.
However, recently it was demonstrated in the visually-hunting predatory Neotropical cichlid species (*Crenicichla frenata*) has most likely the capacity for trichromatic color vision, given that two of the visual pigments of the photoreceptor cells have their absorption maxima at wavelengths of 614 nm and 547 nm respectively (Weadick et al., 2012). Assuming similar visual capabilities for our predator species and in the light of the inferred spectral reflectances presented in Figure 11, this should generously permit the perception of yellowish, gold and orange coloration, and therefore of Midas cichlid and goldfish color morphs.

We are aware of the fact that the current experimental design cannot clarify of how exactly this is mechanistically achieved and see the strength of our study stems in the clear response and its matching with ecological theory and therefore of its putative evolutionary relevance. Reduced crypsis and, therefore, increased vulnerability to predation is most likely the crucial reason of why the gold morphs have been attacked first as a rule by the predator. Possibly, this is also the explanation why in the wild, so few *gold* Midas cichlid morphs occur. Midas cichlids prevail within the structured shore benthic habitat mainly consisting of steep rock faces, jumbled boulders and sunken trees (Barlow, 1976). When being disturbed the fish benefit from this structured habitat in that they hide behind the rock and through background matching in this structured habitat. Further, the *dark* morph only is capable of changing its body coloration quite substantially ranging from pale grey to black. Crucially, this adjustment of body coloration also serves to camouflage the individual against the background. Since the *gold* morph does not dispose of this camouflaging mechanism anymore, *gold* morphs are likely to exhibit a severe disadvantage in terms of an increased predation risk, relatively to the *dark* morph. Indeed, already before entering the transition phase *gold* individuals were reported to exhibit a slower color-matching rate than conspecific *dark* individuals (Dickman et al., 1990).

Despite of putative intraspecific advantages of being *gold* (Barlow, 1973; McKaye & Barlow, 1976; McKaye, 1980) and *gold* being genetically dominant over *dark* (Henning et al., 2010), *gold* Midas cichlid morphs in all natural population of Midas cichlids make up only a small percentage (usually < 10 %) of the adult population (Barlow, 1976; Barlow, 1983). This suggests that being *gold* must come at a cost with respect to natural selection. We believe that our experiment has revealed one such cost. Further, *gold* and *dark* morphs are generally ecologically differentiated (Chapter I) suggesting complex and differential regimes of natural selection acting on
Midas cichlid color morphs in the wild.

In various studies of Midas cichlids from crater lake Xiloá, gold and dark morphs were reported to breed and occur in different depths throughout the lake (Elmer et al., 2009; McKay & Barlow, 1976; McKay, 1980). In a larger context, the idea that predation promotes ecological divergence has gained more and more empirical evidence in recent years (Ingram et al., 2012; Langerhans, 2007; Langerhans et al., 2007; Nosil & Crespi, 2006; Nosil, 2012). Similar, increasing evidence has been accumulated that demonstrates the impact of natural selection via predation on coloration and the associated impact on population structure and ecology (e.g. (Langerhans, 2007; Nosil & Crespi, 2006; Vignieri et al., 2010)). In the light of the herein revealed differential predation pressure, it may be conceivable that predation by aquatic and aerial predators has ultimate ecological implications for the young color-polymorphic adaptive radiation of crater lake cichlids. It is likely, that ecological differentiation between color morph of Midas cichlids actually reflects different macro-habitat use, possibly due to a behavioral response of avoidance of increased predations pressures imposed by visually hunting predators in lower waters by the gold morph.

Another potentially relevant aspect relates to the question why the genetically determined color change occurs late in ontogeny, when the fish are of a certain body size. Interestingly, laboratory-raised Midas cichlids of gold x gold parents (obtained as wild-caught larvae) come into transition at ca. 10 cm of standard length and only rarely earlier (personal observation). Given the result of the present study, it is therefore conceivable that this late ontogenetic onset of color transition partly evolved due to differential predation pressures of Midas cichlid color morphs by aquatic piscivorous predators and their constraints in feeding ecology (mouth gape size). Indeed, average large individuals of Parachromis managuensis in Nicaraguan crater lakes reach ca. 300 mm total length and can be expected to ingest Midas cichlids of at least 10 cm total length (Barlow, 1976); personal observation).

A previous study on differential predation on Midas cichlid color morphs, has led to an opposing result (Annett, 1989). That study focused on largemouth bass, Micropterus salmoides as predator and found that dark Midas cichlids were proportionally more often devoured (69.2 %) than gold morphs (Annett, 1989). We speculate that this is due to substantial differences between our and that previous study. First, a non-native predator was used (largemouth bass, Micropterus salmoides). Second, long-term effects (ca. 4 to 21 days) were addressed rather than instantaneous
foraging decision making. Third, in that study the null hypothesis of equal survival probability of both color morphs is technically only met at the beginning of each trial given the group-wise experimental set up (10-27 individuals per color morph and trial). Providing, prey coloration played a role the predator’s foraging decision in that experiment, the probability of survival of each individual must have changed depending on how many fish of what color have been devoured already. Fourth, the previous experiment (Annett, 1989) was conducted against a uniform background simulating the open water column, whereas in our experiment we aimed at imitating the natural shore habitat from Nicaraguan crater lakes, by using a structured fish tank with the bottom covered with gravel and wood. Also, the inside wall mirrored the interior, therefore increasing complexity. Indeed, Annett (Annett, 1989) admitted “In contrast, piscivores attacking Midas cichlids in aquatic vegetation or in other dark, patterned habitats may be more likely to see the gold morph.”

We assume that the outcome of our laboratory experiment largely applies to the situation in the wild, given the relative rarity of gold morphs of Midas cichlids that are encountered in the wild and the associated prevalence of Parachromis managuensis. However, we see that Parachromis managuensis is not the only naturally occurring predator of Midas cichlid color morphs, although certainly one of the most abundantly occurring in the wild (Barlow, 1976). Fish eating diving birds have been reported to occur abundantly in Nicaragua. This source of predation is also likely to act differentially on Midas cichlid color morphs. Given the fact that birds of prey are visual hunters we expect a similar pattern as has been revealed by aquatic piscine predators in this study. In a field set-up involving dummy fish, we are currently investigating the influence of avian predation in the wild on the Midas cichlid color-dimorphism. Preliminary results reveal that various birds of prey readily attack Midas cichlid dummies of different coloration. These include at least osprey, kingfisher, cormorants and herons.

Although a clear result has been obtained here under controlled laboratory conditions, we cannot exclude the involvement of other described phenomena in impacting the long-term population dynamic structure of color-polymorphic Midas cichlid fishes in the wild, given the role of other involved predators. One particularly interesting example in this regard is negative apostatic (frequency-dependent) selection, through which the dark morph might actually exhibit a higher predation risk than the gold morph. Apostatic selection includes a variety of phenomena (Endler &
Greenwood, 1988), notably search images. Although contested (Crawley, 2009; Guilford & Dawkins, 1987), the formation of search images by predators (Tinbergen, 1960) may explain why color polymorphism are maintained in the wild. The searching image hypothesis (Tinbergen, 1960) applies for visually-hunting predators and implies a changing capability by the predator to perceive different prey types, based on encounter rate, and therefore likely based on frequency in the population. This would result in a disproportional bias to attack the common prey type more often. Through this kind of negative frequency dependent selection (Clarke, 1962) it is conceivable that the rare morph (here in our case the conspicuous gold morph) would gain some relative survival probability, compared to the common (here: dark) morph, potentially overriding the cost of conspicuousness per se (Nosil & Crespi, 2006; Olendorf et al., 2006). Importantly in order to maintain such a color polymorphism over time, the search image should be lost once the previously common prey (here: dark morph) has dropped in abundance and eventually a new search image is formed towards the previously rare (here: gold morph), but now abundant prey (Clarke, 1962; Murdoch et al., 1975).

However, if the population dynamic structure in Midas cichlid populations in the wild were largely driving by predators search image formation, it remains unclear why in none of ten recorded color-polymorphic Midas cichlid populations, gold morphs are actually found more abundantly than dark morphs (Chapter I). Also, the searching image hypothesis is probably most meaningful for cryptically color polymorphic systems (Bond, 2007; Crawley, 2009; Tinbergen, 1960), and other factors have been hypothesized to contribute to the maintenance of prey polymorphism, such as population size and differences in conspicuity between prey morphs (Merilaita, 2006). Given the extreme color differences between Midas cichlid color morphs (Figure 1) and therefore in relative conspicuity, it appears unlikely that even if the search image hypothesis was formed by major Midas cichlid predators, this would compensate for this great discrepancy in crypsis.

The predators readily accepted live fish as part of their regular diet when the experiment started. One might argue that they might have been already conditioned by the display of prey fish and food intake was already anticipated upon the experimenter’s acting at the test tank and that this might have induced a diet preference with respect to particular prey fish colors and eventually due to differences in palatability. We can largely exclude this due to several reasons. First, there was no sign that any of the randomly selected prey fish species or colors were preferred over any other in terms of
hunting motivation and palatability. Second, unlike in the experimental trials, the predators were always fed a single prey fish at a time before participation in the experiment. Third, at the beginning of the experiment the predator had never encountered goldfish before.

Altogether, our study reveals the powerful potential of predation, as a major agent of natural selection, in impacting color-polymorphic populations. We assume that predation has great relevance in the maintenance of one of the most conspicuous color polymorphism in the wild and anticipate that differential predation might contribute to the ecological differentiation between color morphs in the wild, and possibly beyond.

**Acknowledgements**

We are grateful to Peter Buchhauser and the Nicaragua (2012) field work team (C. Fruciano & G. Machado-Schiaffino) for providing Parachromis managuensis. Wild-caught fish were sampled and exported with permission of the Nicaraguan government. Special thanks to S. Stockmaier, H. Recknagel, K. Radtke and J. - P. Morath for their assistance in the lab. We thank R. Schneider and Prof. Michiels from the University of Tübingen for the measurements of spectral reflectance and R. Schneider in particular for his help with the interpretation of the results. We are grateful to F. Korner-Nievergelt and J. Torres-Dowdall for their advice with statistical analysis and experimental design. Funding was provided through various grants of the Deutsche Forschungsgemeinschaft to AM, European Research Council (ERC) advanced grant “GenAdap” to AM and of Deutsche Cichliden Gesellschaft to HK.
Chapter III

Individual specialization in an extremely young adaptive radiation of Nicaraguan crater lake cichlid fishes reflects ecological release

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Under review in *BMC Evolutionary Biology*
Abstract

Background

Individual specialization and frequency-dependent intraspecific competition can play an important role for ecological speciation. Throughout freshwater fishes, adaptive diversification and speciation often occur along the benthic-limnetic axis. A typical pattern of diversification is the independent origin of elongated open water (limnetic) species and of high-bodied shore (benthic) species from generalized ancestors. This has also been demonstrated in the Midas cichlid species complex (*Amphilophus citrinellus* Günther) from Nicaragua, where several volcanic crater lakes have been colonized from an ancestral great lake population. In these new habitats that provide untapped ecological opportunities, new Midas cichlid species have arisen along the benthic-limnetic axis. In the only maximally 1,245 year-old crater lake Asososca Managua, a phenotypically variable Midas cichlid species was recently identified. Here we evaluate this variability and we test for signatures of divergence along the benthic-limnetic axis, as would be expected under adaptive radiation theory.

Results

The investigated Midas cichlid species varied widely and continuously in ecologically-relevant traits. Ranges in stable isotope values spanned multiple trophic levels and the variance exceeded that of whole lake populations of Midas cichlids containing up to six ecologically-differentiated species. Strong phenotype-environment correlations were revealed indicating niche adaptation at the individual level. Neutral genetic differentiation within the lake was not detected based on 13 microsatellite loci.

Conclusions

In this young adaptive radiation of crater lake Midas cichlids both the benthic and the limnetic niche is explored. Divergence and speciation along the benthic-limnetic axis has occurred repeatedly across several taxa of freshwater fishes and is one of the most convincing examples of convergent and parallel evolution. We found a substantial degree of phenotype-environment correlations suggesting individual specialization. It is likely that negative frequency dependent intraspecific competition along with divergent
selection is at work in shaping this population. The involvement of character release, following ecological opportunity, is conceivable, given the comparison with other crater lakes containing benthic-limnetic species pairs. Yet, there is little evidence for a diversifying response to selection in terms of sympatric speciation within the lake, making this a prone system for studying earliest stages of divergence.

**Introduction**

Understanding how biological diversity and intraspecific ecological, morphological and genetic variation is created and maintained is of central interest in evolutionary biology. Ecological disruptive selection is a major evolutionary driving force that promotes biological diversification and that is commonly considered to be an essential cause in the formation of new species (Bolnick & Fitzpatrick, 2007; Nosil, 2012; Schluter, 2000) (termed “ecological speciation” (Nosil, 2012)).

During the process of incipient (ecological) speciation disruptive selection can also be reflected in substantial degrees of individual ecological specialization and intraspecific competition for resources. This should therefore translate into significant phenotype-environment correlations (Martin & Pfennig, 2009; Schluter, 2000). Driven by negative-frequency-dependent processes favouring rare phenotypes, disruptive selection can maintain and even lead to an increase in phenotypic variance, especially when resources vary continuously and the exploration by the consumer affects the relative abundance of the resources (Rueffler et al., 2006; Svanbäck & Bolnick, 2007). In a simplified scenario the fittest individuals are those that explore the most abundant resource category most efficiently, and phenotypically the population should eventually converge on this phenotype (Rueffler et al., 2006). Subsequently, the most abundant resource is depleted and rare phenotypes tend to be favoured. This regime of negative-frequency-dependent divergent selection leads to an increase of phenotypic variation. Similarly, increased individual variation in foraging behavior can cause and trigger the evolution of phenotypic variation and therefore decrease intraspecific competition (Rueffler et al., 2006), as has been suggested for example for three-spine sticklebacks (*Gasterosteus aculeatus*) in postglacial lakes (Bolnick, 2004; Rueffler et al., 2006; Schluter & McPhail, 1992).
Individual specialization is a widespread but underemphasized (Bolnick et al., 2003) phenomenon in nature and indicates the individuals’ restriction of niche exploration relative to the population (Bolnick et al., 2003), most probably due to trade-offs that constrain an individuals’ resource use (e.g. Hatfield & Schluter, 1999). Individual specialization has important eco-evolutionary consequences because the variation in individuals' niche use directly affects the degree of intrapopulation competition and therefore the capacity towards diversification and speciation (Bolnick et al., 2003). It has been proposed (Bolnick et al., 2003; Svanbäck & Bolnick, 2007) that through individual specialization, frequency-dependent processes are facilitated that potentially lead to the broadening of the resource spectrum (Van Valen, 1965), the evolution and maintenance of polymorphisms (Smith & Skúlason, 1996; Swanson et al., 2003) and finally (sympatric) ecological speciation (Bolnick & Fitzpatrick, 2007; Bolnick, 2011; Dieckmann & Doebeli, 1999). Therefore, putative early cases of divergence that are studied at the individual level are the most valid context for analysing incipient events of speciation and adaptive radiation (Bolnick et al., 2003; Bolnick, 2011; Matthews et al., 2010; Schluter, 2000).

**Figure 13. Divergence along the benthic-limnetic axis in Nicaraguan crater lakes.**

In western Nicaragua (Central America), several crater lakes have been colonized independently by Midas cichlids from the great lakes of Nicaragua (Lake Managua and Lake Nicaragua). Midas cichlids in crater lakes Apoyo and Xiloa have speciated along the benthic-limnetic axis. High-bodied „benthic“ specimens are thought to be adapted to the shore region, whereas slender-bodied „limnetic“ individuals live and forage in the open water column. This study focuses on the small and young crater lake Asososca Managua, that houses an endemic and rather limnetic Midas cichlid species (*Amphilophus tolteca*) that is variable along the benthic-limnetic axis.
Instances of parallel evolution, where similar phenotypes arise independently in different environments from a recent common ancestor, provide strong evidence for natural selection in driving diversification (Elmer & Meyer, 2011; Schluter & Nagel, 1995). In freshwater fishes, one major avenue of parallel diversification often takes place along the benthic-limnetic axis (Figure 1, Figure 13) (Barluenga et al., 2006; Elmer et al., 2010a; Jonsson & Jonsson, 2001; Malmquist et al., 1992; McPhail, 1994; Østbye et al., 2006; Robinson & Wilson, 1994; Schluter & McPhail, 1992), where benthic species are characteristically high-bodied whereas limnetic species are of rather fusiform (elongated) body shape (Webb, 1982; Webb, 1984) and these alternative body forms are associated with a benthic (shore-associated) vs. a limnetic (open and deep water) life style. The best studied examples of eco-morphological differentiation involving disruptive selection along the benthic-limnetic axis are fishes in postglacial lakes, such as the three-spine stickleback, *Gasterosteus aculeatus* (e.g. McPhail, 1994; Schluter & McPhail, 1992), *Coregonus* whitefish (e.g. Hudson et al., 2005; Østbye et al., 2006), arctic char, *Salvelinus alpinus* (e.g. Jonsson & Jonsson, 2001; Malmquist et al., 1992) and perch, *Perca fluviatilis* (e.g. Svanbäck & Eklöv, 2003) that have diversified, often in multiple independent instances, into benthic and limnetic forms.

It has been shown that Neotropical Midas cichlids (*Amphilophus citrinellus* Günther) from Nicaraguan great lakes and crater lakes also mirror this pattern of sympatric differentiation (Barluenga et al., 2006; Elmer et al., 2010a; Meyer, 1990a; Meyer, 1990b). The Midas cichlid species flock has become a model system for studying adaptive radiation and incipient speciation in parallel mostly along the benthic-limnetic axis as well. In western Nicaragua, several crater lakes have been formed by accumulation of rain and ground water in isolated volcanic calderas. In rare and likely independent events, Midas cichlid generalist ancestors from the great Nicaraguan lakes colonized these newly formed crater lakes and have diversified sympatrically along the benthic-limnetic axis while exploiting niches uniquely available in the crater lakes (Barluenga et al., 2006; Elmer et al., 2010a; Elmer et al., 2013). This sympatric differentiation along the benthic-limnetic axis is consistent with theory and model predictions (Elmer et al., 2010a; Gavrilets et al., 2007). Clearly, in some crater lakes Midas cichlids have speciated along the benthic-limnetic axis (e.g. in lakes Apoyo and Xiloá) endemic limnetic species were formed such as *A. zaliosus* and *A. sagittae*,

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respectively), but how this differentiation was achieved ecologically remains largely unexplored and requires examining individuals within variable populations that have not yet speciated to quantify the individual specialization that may eventually promote speciation through disruptive selection (Bolnick et al., 2003; Bolnick, 2011; Martin & Pfennig, 2009; Smith & Skúlason, 1996; Swanson et al., 2003).

We are here addressing these issues of individual specialization and divergence along the benthic-limnetic axis in an extremely young crater lake species of Midas cichlids, *Amphilophus tolteca*, that is a suitable model system in which to study the early stages of divergence by disruptive selection. *Amphilophus tolteca*, an overall rather limnetic Midas cichlid species (Recknagel et al., 2013) endemic to the maximally 1,245 year old (Pardo et al., 2008) crater lake Asososca Managua that appeared being phenotypically remarkably variable along the benthic-limnetic axis. We investigated the extent of individual specialization, as well as associated phenotype-diet correlations and relative ranges of long-term signatures of diet, indicated by stable isotopes nitrogen (δ¹⁵N) and carbon (δ¹³C). It has been suggested, though never tested, that this population has differentiated into macro-habitats, and that discrete morphs (or even species) might have evolved within such a short period of time (Elmer et al., 2010a). We comprehensively tested this by assessing genetic clustering and trait bimodality in relevant body morphology along the benthic-limnetic axis (Body-Height Index [BHI], see Methods section for definition), body and lower pharyngeal jaw (LPJ) size and shape, and stable isotope signatures. We found strong evidence for individual specialization and revealed strong phenotype-diet correlations, crucial ingredients for frequency-dependent evolutionary processes, and subsequently disruptive selection (Bolnick, 2011). Finally, we demonstrate that discrete morphs/species have not (yet) evolved in this evolutionary young population of Midas cichlids.

**Material and Methods**

**Specimen collection**

*Amphilophus tolteca* specimens (n = 190) from Asososca Managua (N 12°08.390’ W 086°18.792’) were collected by gill-nets or harpooning in 2010 and 2012. In the field, standardized photographs that include a ruler were taken from directly above using a
tripod and a Canon Power Shot D10 digital camera. Tissue samples (fin, muscle) were collected and stored in pure ethanol. All specimens were taken as vouchers (head or whole body) and stored in 70 per cent ethanol.

This is partly a comparative study on ecotype differentiation along the benthic-limnetic axis. Therefore, already available, but unpublished data on body shape (HK) from the endemic Midas cichlid communities of lakes Apoyo (six species; 488 specimens) and Xiloá (four species; 460 specimens) and Asososca Managua (96 specimens) were consulted. From lakes Apoyo and Xiloá, parallel evolution and speciation along the benthic-limnetic axis has been reported in multiple instances (Elmer et al., 2010a; Elmer & Meyer, 2011). Conventionally, only Amphilophus zaliosus from Lake Apoyo (Barlow & Munsey, 1976) and A. sagittae from Lake Xiloá (Stauffer Jr & McKay, 2002) are classified as limnetic species (Elmer et al., 2010a), whereas the other Midas cichlid species are rather attributed to the benthic habitat. Accordingly, all specimens from these lakes were classified as either limnetic or benthic ecotypes and the species level was ignored. Consequently, “benthic” Midas cichlids from lakes Apoyo and Xiloá comprise representatives of up to five and four species, respectively. Fish were sampled randomly in the field.

**Body shape analyses and definition of Body-Height Index**

Body shape differentiation was analysed using geometric morphometric approaches. Eighteen landmarks (LM) describing body shape were digitized from photographs of 1,138 individuals in tpsDig v. 2.16 (Rohlf, 2010a) by a single investigator (HK; Figure 14, landmarks modified from Manousaki et al., 2013). Shape analyses were performed in MorphoJ1.05a (Klingenberg, 2011), for detailed methods see Elmer et al., 2010a. Body shape data showed significant allometric effects (6.12 % of shape was explained by centroid size; \( p < 0.0001 \)), thus the size-corrected shape data (regression residuals) were considered as variable of interest. Variation in individual body shapes within and across lakes was visualized using principal component analysis (PCA). The thin plate spline technique (Dryden & Mardia, 1998) was used to visualize body shape differences between groups as well as shape changes associated with principal components (PCs).

Further, we defined the Body-Height Index (BHI) to quantitatively assign each specimen along the benthic-limnetic axis with respect to body shape. BHI is the relative
fraction of body height as a function of standard length. BHI was calculated from inter-landmark distances that were obtained in PAST v. 2.16 (Hammer et al., 2001) for each individual. The inter-landmark distance between LM 6 and LM 9 (Figure 14) was used as a proxy for body height.

Figure 14. Benthic/limnetic differentiation: Definition of eco-morphological measurements.
Left side: Anatomical description of body landmarks: LM 1 = tip of snout; LM 2 = most posterior point of the lips; LM 3, 4 = most anterior and posterior margin of the eye; LM 5 = intersection of operculum and ventral body outline; LM 6 = anterior base of dorsal fin; LM 7, 8 = dorsal and ventral base of pectoral fin; LM 9 = anterior base of pelvic fin; LM 10, 11 = anterior and posterior base of anal fin; LM 12 = posterior base of dorsal fin; LM 13, 14 = dorsal and ventral base of caudal fin; LM 15 = midpoint of the caudal fin origin; LM 16 = nostril; LM 17 = dorsal end of preoperculum; LM 18 = dorsocaudal origin of the operculum. Right side: Anatomical description of LPJ landmarks and caliper measurements: LM 1, 2 & LM 10, 11 = tips of posterior and lateral processes of upper horn; LM 3, 9 = points of highest curvature in upper horn base; LM 4, 8 = points of closest intersection between horn base and dentition area; LM 5, 7 = points of highest curvature near the mid-point of the two adjacent landmarks; LM 6 = posterior-most point of lower pharyngeal jaw suture; LM 12-15 & 18-21 = points of highest curvature near the mid-point of the two adjacent landmarks; LM 16, 17 = anterior tips of lower horn processes; LM 22, 23 = most posterior teeth of the “external line” of the dentition area; LM 24 = point where the suture meets the dentition area. Caliper measurements LPJ width, length and depths are also illustrated (grey arrows). Jaw and body shape outlines modified from Manousaki et al., 2013.
Lower pharyngeal jaw shape analyses

Lower pharyngeal jaws (LPJ) were extracted from the head of 96 ethanol-preserved *A. tolteca* specimens. External characteristic features of 96 LPJ (lateral horn width, jaw length, keel depth; Figure 14) were measured using a digital caliper and jaws were weighted to the nearest mg using a precision scale. In a complementary approach, standardized photographs from were taken from the extracted and cleaned LPJ. Jaws were placed as plane as possible in an agarose gel chamber and the dentition side was photographed from directly above using a tripod and a Canon Power Shot D10 digital camera. 24 homologous landmarks, consisting of 12 fixed and 12 semi-landmarks were defined that describe external LPJ shape as well as the shape of the dentigerous area. Digitization was done in tpsDig v. 2.16 (Rohlf, 2010a) by a single investigator (HK) from the photograph of each specimen (Figure 14). Semi-landmarks were slid in tpsRelw v. 1.49 (Rohlf, 2010b) in orthogonal projection mode with 10 iterations. Slid semi-landmarks were treated as true homologous landmarks in MorphoJ1.05a (Klingenberg, 2011). Object symmetry was taken into account and the symmetric component of shape variation only was considered as our trait of interest (Klingenberg et al., 2002). A correction for allometric effect on LPJ shape was performed by regressing Procrustes coordinates on centroid size (12.47 % explained; \( p < 0.0001 \)). Regression residuals were used in downstream analyses that were conducted analogous to body shape analyses.

Long-term analysis of diet: inferences from stable isotopes

Stable carbon (\( \delta^{13} \text{C} \)) and nitrogen (\( \delta^{15} \text{N} \)) isotope ratios were used to infer trophic divergence along the benthic-limnetic axis. Generally, \( \delta^{13} \text{C} \) indicates the relative source of benthic and limnetic carbon and thus presumably macro-habitat and \( \delta^{15} \text{N} \) indicates the relative trophic level (Bolnick, 2011; France, 1995; Hecky & Hesslein, 1995; Post, 2002; Zanden et al., 1999; Zanden & Rasmussen, 1999). A small piece of muscle tissue was extracted from dorsal musculature of 74 ethanol preserved specimen of *A. tolteca* and dried for ca. 48 hours at 55°C. Samples were ground in individual sealed tubes and a 1-1.5 mg subsample was weighted. Analyses were done by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) at the Limnological Institute (University of Konstanz). \( \delta^{13} \text{C} \)-values were corrected for lipid content.
In R (R Core Team, 2012) Levene’s test was used (Levene, 1960) to assess any differences in isotopic variances between i) *A. tolteca* and pooled Midas cichlid populations from lakes Apoyo and Xiloá and ii) between *A. tolteca* and limnetic species of lakes Apoyo and Xiloá only.

**Assessments of individual ecological specialization in Asososca Managua**

To assess significant interactions of diet and eco-morphological variables at the individual’s level, linear regression analyses were conducted in R (R Core Team, 2012) to test for individual correlations of morphology and the carbon-source and the trophic level as indicated by nitrogen (Post, 2002). Stable isotope signatures were tested for correlation with LPJ weight ($n = 54$) and BHI ($n = 73$). Aiming at detecting correlation of jaw- and overall body morphology, BHI and LPJ weight ($n = 95$) were tested. LPJ weight was selected, because it highly correlated with the other LPJ variables (e.g. see Figure S6), which we therefore consider as overall index of jaw hypertrophy. BHI was used because it is highly correlated with PC1-3 of the body shape analysis and because it is a clear indicator of body elongation (and thus individual specialization along the benthic-limnetic axis; see also Figure S6).

Aiming at testing for indicators of incipient speciation, we assessed the nature of the trait distributions in our sample. The following measurements were combined into one data frame in R (R Core Team, 2012) that comprised information of 53 individuals: individuals scores of PCs of the intralacustrine body and LPJ shape analyses, BHI, allometry-corrected LPJ-variables weight, depth, width and length as well as both stable isotope signatures $\delta^{13}$C and $\delta^{15}$N. A model based clustering approach was applied to the above mentioned combined and standardized data set of 53 individuals using the mclust package (Fraley et al., 2012). This Bayesian approach was used to identify the optimal model among a range of parameterized Gaussian mixture models for 1-9 clusters and varying covariance matrices.

Further, in a complementary approach largely following (Kusche et al., 2012 - Chapter IV of the present thesis) we conducted a mixture analysis on all variables to determine whether they most likely consisted of one or two single-component normal distributions. This line of inquiry comprises a number of statistical tests to evaluate the presence of discontinuous variation in trait distributions. If divergent selection was at
work in driving these fish apart along the benthic-limnetic axis, then signatures of selection should be identifiable when trait distributions are analyzed (Rundle & Nosil, 2005; Schluter, 2000). The mixture analysis comprised a test for unimodality (dip test (Hartigan & Hartigan, 1985)), a test for platykurtosis (Anscombe-Glynn test (Anscombe & Glynn, 1983)) and an expectation–maximization (EM) algorithm-based approach (McLachlan & Peel, 2000) implemented in the mixtools package (Benaglia et al., 2009). In the latter, mixtures of two single-component normal distributions were fitted to data set and the fit was inspected. A parametric bootstrap test (1000 iterations) using the output of the EM mixture model fits was performed to test whether the trait distributions consisted of one or two components (Benaglia et al., 2009). Normal probability plots and Shapiro-Wilk tests (Shapiro & Wilk, 1965) were also consulted to infer whether a particular data set deviated from a single normal distribution.

Assessment of neutral genetic differentiation

Considering previously published conditions, 13 microsatellite loci were amplified and scored using the same methods as in our previous publications for 118 individuals from Asososca Managua (M1M, M2, M7, M12 (Noack et al., 2000), UNH002 (Kellogg et al., 1995), UNH011, UNH012, UNH013 (McKaye et al., 2002), Abur45, Abur82, Abur151 (Sanetra et al., 2009), Burtkit F 474/R672 (Salzburger et al., 2007), TmoM7 (Zardoya et al., 1996)).

Descriptive statistics such as the inbreeding coefficient $F_{is}$ and gene diversity were calculated based on the FSTAT vers. 2.9.3.2 (Goudet, 1995) output file. Rarefied allelic richness was assessed in HP–Rare vers. June-6-2006 (Kalinowski, 2005).

Structure vers. 2.3.3 (Pritchard et al., 2000) was used to determine genetic differentiation within this population of Midas cichlids following (Evanno et al., 2005). The setting parameters comprised 50 000 burnin periods, 500 000 repetitions (Falush et al., 2003) and k=1-5.
Results

Ecological and genetic variation along the benthic-limnetic axis

In this study eco-morphologically important traits such as body shape and lower pharyngeal jaw (LPJ) shape as well as long-term signatures of diet were investigated. In *Amphilophus tolteca* these traits were found to be highly variable along the benthic-limnetic axis (Figures 13 & 15). In contrast, the within population genetic variation, as inferred from 13 microsatellites, was only moderate (Table S4).

Body shape

Body shape is an overall indicator of habitat preference and thereby major ecological niche use along the benthic-limnetic axis. The principal components biplot reflected a substantial diversity of body shape phenotypes in the population (Figure 15A). These ranged from typically benthic high-bodied individuals with relatively small heads to typically limnetic individuals of substantially elongated body shape. Rather benthic fish were mainly characterized by a dorso-ventral expansion and anterior-posterior contraction of the transformation grid, most importantly involving landmarks (LM) 6 and LM 9-12 and a relative shortening of the caudal peduncle (LM 13-15). Accordingly, limnetic-like fish were mainly characterized by a dorso-ventral compression of the grid, a relative elongation of the caudal peduncle and the head (LM 1-5, 7-8, 17-18), compared to benthic-like fish.
Figure 15. Midas cichlid variation in Asososca Managua along the benthic-limnetic axis.
A) Body shape: Biplots of PCs 2 and 3 that encode for 24.67 \% of the total body shape variation. The line terminus in the transformation grids depicts the shape changes from the overall mean associated with each PC (scale factor of 0.1 in positive and negative
directions). Positive values on PCs 2 and 3 indicate rather high-bodied (benthic-like) individuals, whereas negative values on both PCs overall indicate rather elongated (limnetic-like) individuals. See Figure 14 for detailed landmark definitions. B) LPJ shape. Biplots of PCs 1 and 2 that encode for 45.78% of the total LPJ shape variation. Deformation grids depict a scale factor of 0.1 in positive and negative directions on both PCs. Positive values on PC1 and negative values on PC2 indicate rather delicate LPJ-morphologies („papilliform”) individuals. See Figure 14 for landmark definitions. C) Biplots of stable isotopes $\delta^{15}$N and $\delta^{13}$C. The more enriched an individual is in $\delta^{15}$N, the higher is its trophic level. The bar indicates the range of $\delta^{15}$N that is conventionally-defined as a single trophic level. $\delta^{13}$C is usually enriched in individuals, that preferentially forage in the benthic habitat and depleted in limnetic ecotypes.

**Lower Pharyngeal Jaw shape**

LPJ morphology likely reflects an individual's feeding history and is therefore an indicator of preferential niche use. This is because the teeth are replaced regularly and respond with pronounced phenotypic plasticity (Muschick et al., 2011). The LPJ morphology varies between a pronounced “molariform” sturdy type of pharyngeal jaw that is designed to crack hard diets such as snails found in these crater lakes – to a “papilliform” morphology with more slender bones and teeth (Meyer, 1990a; Meyer, 1990b). There is performance trade-off where molariform LPJ allow the cracking and processing of hard snail shells, but those individuals are less efficient in processing softer prey items and *vice versa* (Meyer, 1989). Within the Midas cichlid population of Asososca Managua, a range of LPJ-morphologies were detected (Figure 15B) that varied along the benthic-limnetic axis. PC1 and PC2 captured 26.02% and 19.76% of the total variation in LPJ shape, respectively. Positive PC1 scores reflected jaws that had overall long, but not necessarily sturdy, horns. PC2 was a good indicator of the overall LPJ morphology, with positive values representing LPJ margins warped towards the outside (LM 4-8, 12-14 and LM 19-21) as well as wider and sturdier lateral horns (LM 1-4, LM 21 and LM 8-12). Also the dentition area (as defined by LM 3-8 and LM 22-24) was relatively enlarged. This robust LPJ morphology has been typically termed “molariform” in contrast to the relatively delicate, usually small-toothed “papilliform” LPJ type; e.g. (Meyer, 1990a; Meyer, 1990b), which was mainly associated with negative values on PC2.
Long-term analysis of diet: inferences from stable isotopes

The long-term analysis of diet revealed a large variance in stable isotope signatures within Asososca Managua Midas cichlids (Figure 15C). The $\delta^{15}$N signatures ranged from 11.62 ‰ to 18.33 ‰ (Δ 6.71 ‰) and the $\delta^{13}$C signatures ranged from -33.37 ‰ to -25.82 ‰ (Δ 7.55 ‰), indicating high levels of consistent ecological resource use and thus individual foraging specialization.

Neutral genetic markers

A relatively low level of polymorphism was identified in the multilocus analysis of 13 polymorphic microsatellites. The number of alleles per locus ranged between 2 (Abur 151) and 14 (M7 & UNH013) (Table S4). The inbreeding coefficient ($F_{\text{is}}$) was 0.051. Gene diversity was 0.549 and allelic richness was 7.28. Our new and more extensive microsatellite data are largely congruent with a previous genetic report (Barluenga & Meyer, 2010) on this population of Midas cichlids.

Relationships between diet and eco-morphological traits

Individual specialization is likely when morphological adaptations exhibit ecological relevance. We here present strong evidence for such correlations. Overall, we found that the relevant body morphology along the benthic-limnetic axis (as indicated by Body-Height Index [BHI]) largely corresponded to overall jaw morphology (LPJ weight), which itself highly reflected feeding ecology as measured by stable isotope signatures (Figure 16). Specifically, BHI and LPJ weight were found to be positively correlated (linear model: $r = 0.24$, $n = 95$, $p < 0.05$). LPJ weight was negatively correlated with the isotope predominantly indicating trophic level, $\delta^{15}$N, ($r = -0.33$, $n = 54$, $p < 0.05$) and showed strong positive correlations with the carbon source, $\delta^{13}$C, that typically characterizes preferential use of benthic-limnetic macro-habitats (Post, 2002) ($r = 0.48$, $n = 54$, $p < 0.001$). BHI generally showed the same trend as LPJ weight did, although the effect was overall less significant ($\delta^{15}$N: $r = -0.09$, $n = 73$, not significant; $\delta^{13}$C ($r = 0.24$, $n = 73$, $p < 0.05$) (Figure 16).
Figure 16. Correlogram of eco-morphological and diet variables.
Pair-wise correlations of the eco-morphological variables BHI, LPJ weight, $\delta^{15}N$, and $\delta^{13}C$ are depicted together with the outcome of linear regression analysis. The solid line indicates the regression line. The 95% confidence interval is indicated by the dotted lines. BHI was positively correlated with LPJ weight and $\delta^{13}C$. LPJ weight was negatively correlated with $\delta^{15}N$ and positively correlated with $\delta^{13}C$. The two stable isotopes are negatively correlated. Limnetic-like individuals (indicated by low BHI values) tend to feed on a slightly higher trophic level (enriched in $\delta^{15}N$) than benthic-like individuals (high BHI values). Further, elongated fish tend to be less enriched in $\delta^{13}C$, indicative of their pelagic lifestyle.
This combined analysis reflected both ecological divergence within *A. tolteca* in Asososca Managua as well as a substantial degree of individual specialization. Fishes with elongated body shapes (low BHI values) tended to feed on a slightly higher trophic level (enriched in $\delta^{15}N$) as is predicted in limnetic ecotypes relative to high-bodied benthic individuals (Post, 2002; Zanden et al., 1999; Zanden & Rasmussen, 1999). Further, these elongated fish tended to be less enriched in $\delta^{13}C$, most probably indicating their pelagic life style (France, 1995; Hecky & Hesslein, 1995; Matthews et al., 2010; Post, 2002; Zanden et al., 1999; Zanden & Rasmussen, 1999). Fish caught in the open water of As. Managua are substantially more elongated than fish caught in the shore region (Meyer et al.; personal observation). Overall, diet as inferred from stable isotopes $\delta^{13}C$ and $\delta^{15}N$ was consistently correlated with morphological traits affecting directly feeding ecology, especially LPJ weight.

**Across-lake comparison of eco-morphology and isotopes**

To evaluate the degree of divergence in the Midas cichlids of Asososca Managua, we contrasted the patterns of ecological differentiation between the Asososca Managua population and those from the older crater lakes Apoyo (about 24,000 years) and Xiloá, (about 6,000 years) that both house multiple endemic divergent species along the benthic-limnetic axis (Elmer et al., 2010a).

**Body shape and Body-Height Index**

The joint principal component analysis (PCA) of specimens from Asososca Managua, Apoyo and Xiloá revealed partial overlap of *A. tolteca* with benthic and limnetic specimens from lakes Apoyo (6 species) and Xiloá (4 species) (Figure 17). PC1 and PC3 together accounted for 38.41% of the total variation and represented a change in body elongation, body height and relatively more posterior dorsal and anal fin placement (Figure 17). These were body shape features that are typically known to vary along the benthic-limnetic axis ((Elmer et al., 2010a); see also “arrow shaft metaphor” that describes the body shape of limnetic cichlids in (Fryer & Iles, 1972)).
Figure 17. Midas cichlid body shape differentiation along the benthic-limnetic axis. Depicted are 90% confidence ellipses from the biplot of PCs 1 and 3 obtained from the joint principal component analysis of body shape of lakes Apoyo, Xiloá and Asososca Managua. The differentiation between ecotypes from lakes Xiloá and Apoyo is more pronounced, compared to Asososca Managua. Shape changes along PCs 1 and 3 (scale factor = 0.1) are indicated by thin plate splines. The line terminus refers to the shape change along a particular principal component, compared with the average shape (black dot). The shape changes along PCs 1 and 3 correspond to typical body shape differences along the benthic-limnetic axis (relative body height and elongation as well as snout bluntness). Positive values on PC1 and negative values on PC3 indicate rather benthic individuals. Groups are color-coded.

The Body-Height Index (BHI), is a summary statistic of body shape where lower values mean greater elongation and therefore shallower bodies, revealed that many individuals of *A. tolteca* were substantially elongated and the average Midas cichlid from Asososca Managua was more elongated (0.41 ± 0.02, range: 0.361 to 0.485) than the average individual from lakes Apoyo (6 species; 0.427 ± 0.04, range: 0.313 to 0.518) or Xiloá (4 species; 0.449 ± 0.03, range: 0.367 to 0.535) (Figure 18A). Some *A. tolteca* individuals
showed similar levels of body elongation as some limnetic species of the endemic Midas cichlids from lakes Apoyo and Xiloá do, but did not reach the extreme BHI values as high-bodied individuals that are characteristic of endemic benthic species from lakes Apoyo and Xiloá (Figure 18A). BHI conformed to a bimodal distribution in lakes Apoyo and Xiloá that both house multiple species, but not in Asososca Managua (Apoyo: \( n = 488, p < 0.001 \); Xiloá: \( n = 460, p < 0.004 \); Asososca Managua: \( n = 286, p = 0.33 \); see also Figure 18B for visualization).

**Figure 18. Differentiation in Body-Height Index across lakes.**
A) Histograms of BHI distribution for Midas cichlid populations of lakes Apoyo, Xiloá and Asososca Managua indicating range and distribution of this body shape summary statistics. B) Normal probability plots with cumulative proportions of observed versus expected proportions of BHI in comparison to a single normal distribution (red line) for lakes Apoyo, Xiloá and Asososca Managua. Trait distributions for lakes Apoyo and Xiloá are best explained by two components and the one of Asososca Managua by only one. Statistical outcomes are described in the “Results” section.

**Stable isotope signatures**
We tested whether the Midas cichlids of Asososca Managua explored a relatively wider ecological space than the other known limnetic Midas cichlid species as well as whole lake populations in those lakes that underwent multiple speciation events along the benthic-limnetic axis.

The variance in the trophic level of *A. tolteca* (indicated by \( \delta^{15}N \)) exceeded that of a randomly drawn sample of lakes Apoyo and Xiloá that house six and four Midas cichlid species, respectively. However, this difference was not statistically significant (Table S5). Interestingly, when the carbon source \( \delta^{13}C \) was considered, the variance across *A. tolteca* was about fourfold larger than that of the limnetic species from either lake Apoyo (*A. zaliosus*) or Xiloá (*A. sagittae*) (both comparisons at \( p < 0.01 \);
Additional file 3). *A. toleca* generally exhibited a higher variance in $\delta^{15}$N than either other limnetic species (comparison highly significant with *A. sagittae* (Lake Xiloá) at $p < 0.001$; Table S5).

**Phenotypic differentiation by disruptive selection**

The high correlation between eco-morphological traits and ecological niche use suggested a benthic-limnetic divergence of Midas cichlids within Asososca Managua. To assess whether this is supported by bimodal eco-morphological trait distributions, as required for speciation (Elmer et al., 2010b; Rueffler et al., 2006), we tested whether the trait distributions were composed of one or two components most likely and whether there was clustering in the data.

The combined mixture analysis without *a priori* specimen classification revealed that the distribution of each ecologically-relevant trait (LPJ measurements, stable isotopes and BHI) was best explained by a single component rather than two components (i.e. unimodality cannot be rejected for bimodality) (Table 2). However, some data sets indicated trends towards (weak) bimodality because they were either marginally non-significant when tested for platykurtosis (a broadened/flattened normal distribution) or normality was only close to being rejected statistically or both (e.g. LPJ width and $\delta^{15}$N or both; Table 2, Figure S7), potentially indicating incipient signatures of diversification in these traits.

When all available variables were combined (jaw caliper measurements + LPJ weight, PCs_{body}, PCs_{jaw}, stable isotopes, BHI) for the maximum subset with complete sampling per individual, one spherical, multivariate cluster was most likely (log-likelihood = -4482, $n = 53$, df = 61, BIC = -9206). The Bayesian clustering approach implemented in the software Structure revealed no support for genetic clustering within the Asososca Managua Midas cichlid population (k = 1: ln = -3369 $\pm$ 0.3; k = 2: ln = -3372 $\pm$1).
Table 2. Testing for bimodality in ecologically-relevant traits within *A. tolteca*.

The analyses included dip tests for unimodality, Anscombe-Glynn tests for platykurtosis, Shapiro-Wilk tests for normality and mixture analysis to determine the number of components a particular data set. All traits were rather supported by continuous distributions.

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Discussion

In this study we focused on the young Midas cichlid flock of the crater lake Asososca Managua that is only maximally about 1,200 years old. In spite of this extremely young age in the youngest of all crater lakes of Nicaragua, we detected surprisingly high degrees of among-individual eco-morphological variation and resource partitioning, demonstrating a substantial degree of individual specialization. We uncovered strong phenotype-diet correlations, thus making the variation in resource use likely subject to frequency-dependent competition (Bolnick, 2011). Altogether, this may provide important raw material upon which disruptive selection can act and ultimately promote intralacustrine divergence via sympatric ecological speciation (Bolnick, 2011). This young crater lake Midas cichlid species explores a relatively wider ecological space than the older (limnetic) Midas cichlid communities in other lakes that have completed speciation along the benthic-limnetic axis, possibly indicating ongoing ecological character release in response to ecological opportunity. However, we could not detect strong responses to disruptive selection, in the sense of incipient diversification along the benthic-limnetic axis, as indicated by lacking bimodality in ecologically-relevant traits. This might indicate that once a novel environment is colonized, and based on ecological opportunities, a species will rapidly diversify phenotypically. Only in a second step during incipient speciation will reproductive isolation evolve permitting for genetic differences to build up. We believe that in the case of the crater lake Asososca Managua we are witnesses for this first stage during this incipient speciation process.

Variability and individual specialization along the benthic-limnetic axis

Our study revealed considerable amounts of within-population variation and diversification in ecologically-relevant traits such as body shape and LPJ-related traits as well as stable isotope signatures that indicate among-individual variation in consistent ecological resource use and therefore individual specialization (Bolnick, 2011). This variation is likely important in the efficient exploration of benthic and limnetic macrohabitats, e.g. (Schluter & McPhail, 1992). In Asososca Managua, body shape phenotypes closely resembled benthic and limnetic Midas cichlid species of other crater lakes (Figure 15A, Figure 17, Figure 18A). Similarly, the variation in LPJ
morphism ranges from delicate (papilliform) to rather robust and sturdy (molariform) LPJs (Figure 15B).

The stable isotope signatures (Figure 15C) in this single population spanned multiple trophic levels ($\delta^{15}$N) according to stable isotope foraging theory (Post, 2002; Zanden et al., 1999; Zanden & Rasmussen, 1999) and suggested the exploration of different (macro)-habitats ($\delta^{13}$C) within this lake (France, 1995; Hecky & Hesslein, 1995; Post, 2002; Zanden et al., 1999; Zanden & Rasmussen, 1999). It is generally assumed that enrichments of $\sim$3 ‰ in $\delta^{15}$N correspond to the shift of a single trophic level, i.e. these differences would be expected in a consumer prey relationship (Post, 2002; Zanden et al., 1999; Zanden & Rasmussen, 1999), whereas enrichments of $\delta^{13}$C would be less important in this regard (0-1 ‰ enrichment along a single trophic level). However, even within a single body of water, $\delta^{13}$C indicates the primary producers that a consumer feeds on, since benthic algae are typically enriched in $\delta^{13}$C relative to free floating phytoplankton of the open water column (France, 1995; Hecky & Hesslein, 1995; Post, 2002; Zanden et al., 1999; Zanden & Rasmussen, 1999). Our data suggest that A. tolteca covers at least two trophic levels and reveals remarkable variation in the carbon source of its prey items along the benthic-limnetic axis. This suggests that some individuals tend to consume predominantly benthic prey items such as snails and presumably Aufwuchs and others tend to consume limnetic prey items such as insect larvae and fish. However, many specimens appear to utilize opportunistically both prey categories. To reconstruct an even more precise image of diet history, future studies might include the isotopic trophic position of A. tolteca prey items in combination with stomach content analysis.

More direct evidence for individual specialization stems from our demonstration of ecological relevance of individual morphologies (Figure 16). We showed that the correlation of morphological and ecological features of individuals conforms to what is typically associated with restricted habitat use along the benthic-limnetic axis (France, 1995; Hecky & Hesslein, 1995; Post, 2002; Robinson & Wilson, 1994; Robinson & Schluter, 2000; Taylor, 1999; Webb, 1982; Webb, 1984; Zanden et al., 1999; Zanden & Rasmussen, 1999), suggesting local adaptation to contrasting macro-habitats. Consistently, benthic-like fish were higher-bodied and less arrow-like-shaped, had more robust pharyngeal jaws, fed at a lower trophic level and exhibited a littoral carbon
source (Figure 16, Figure S6) than limnetic-like fish. In other words, the difference in diet is significantly associated with eco-morphological traits, most importantly with LPJ weight and overall body elongation (BHI). LPJ weight, as a proxy for LPJ hypertrophy, was correlated with long-term diet in terms of stable isotope signatures (Figure 16, Figure S6). This means that the heavier a LPJ of a given individual is, the less enriched this individual will be in $\delta^{15}$N and the more likely its carbon source will be of a benthic origin. This is an important consideration since the robustness of the LPJ limits the food sources a fish can explore, i.e. snail shell crushing requires rather robust and sturdy LPJs (Keenleyside, 1991; Meyer, 1989; Meyer, 1990a; Meyer, 1990b) and snails are substrate-associated, thus restricted to the benthic habitat. LPJ morphology is closely associated with head and body shape in Midas cichlids from Asososca Managua (Figure 16, Figure S6). Indeed, it appears that throughout the species complex, benthic Midas cichlid species consistently tend to have thicker and more robust horns than their limnetic counterparts ((Barluenga et al., 2006; Meyer, 1990a).

These and other ecologically-relevant traits (such as enlarged lips) should be responsive to disruptive natural selection, given their parallel evolution across lakes and demonstrated partial genetic basis (Elmer et al., 2010a; Lu & Bernatchez, 1999; Manousaki et al., 2013; Peichel et al., 2001). However, given the ability in Midas cichlids to plastically respond to different environments with respect to jaw morphology (Muschick et al., 2011), adaptive phenotypic plasticity is likely to have played a considerable role in shaping the documented eco-morphological variance.

**Evolutionary context**

**Ecological opportunity and character release**

Intraspecific competition is considered a diversifying force that creates differences within species (character release), when competing species are absent (Cody, 1974; Robinson & Wilson, 1994; Yeaton & Cody, 1974). Such competition can promote increased individual ecological specialization and resource-specific fitness, which both provide an important framework for frequency-dependent diversification processes promoted by disruptive selection (Bolnick et al., 2003; Robinson & Wilson, 1994).

In the light of our results presented above, one might therefore argue that character release in Asososca Managua following ecological opportunity is
driving/permitting phenotypic diversification (Nosil & Reimchen, 2005; Schluter, 2000). Character release refers to geographical settings where a species occupies a greater range of habitats and phenotypes, than it would if it co-occurred with a similar species (Brown & Wilson, 1956; Grant, 1972; Robinson & Wilson, 1994). In the setting of Asososca Managua, an important consideration in this regard is the relative scarcity of other fish species, not only Midas cichlids, in this lake. Apart from A. tolteca, there are only three other fish species in the lake. Lake Apoyo houses six Midas cichlid and five other fish species and in lake Xiloá, four Midas cichlid species and 14 other species are known to occur (numbers based on (Elmer et al., 2010a; updated following Recknagel et al., 2013).

Amphilophus tolteca appears to cover an ecological space that in other crater lakes is occupied by several ecologically well-differentiated Midas cichlid species along the benthic-limnetic axis, for example morphologically (Figure 17) and especially in terms of variances in stable isotope signatures (Table S5). From an isotopic point of view, we inferred that a range of benthic-limnetic relevant macro-habitats is likely to be explored by A. tolteca (Figure 15C) and that its variance in both isotopes is unusually high, both, compared to the other limnetic species of lakes Apoyo and Xiloá and even to the pooled lake populations with multiple species of Midas cichlids (Table S5). Phenotypically, we revealed substantial overlap in body shape variation between A. tolteca and benthic and limnetic species from lakes Apoyo and Xiloá (Figure 17) suggesting that this species explores a similar range of ecological space along the benthic-limnetic axis as multiple Midas cichlid species from other crater lakes do. The body shape changes associated with PCs 1 and 3 from the across-lake-PCA mainly reflect body elongation and bulkiness of the head region (Figure 17). Relevantly, these eco-morphological features can generally be interpreted as adaptations along the benthic-limnetic axis in cichlids and throughout other adaptive radiations of teleost fishes (Clabaut et al., 2007; Cooper et al., 2010; Elmer et al., 2010a; Jonsson & Jonsson, 2001; McPhail, 1994; Østbye et al., 2006; Robinson & Wilson, 1994; Schluter & McPhail, 1992).

If character release was at work, then it might facilitate the exploration of a range of niches unique to crater lakes, and consequently provide divergent selection forces with an adequate starting material for ecological speciation. We believe therefore
that the situation that we are describing here for the youngest of all crater lakes in Nicaragua represents the first steps along the speciation processes that have led to the origin of four endemic species in the older crater lakes Nicaragua’s such as Xiloá (that is about five times older) and even six endemic Midas cichlid species in the case of crater lake Apoyo (that is almost 20 times older) than Asososca Managua.

**Progress towards sympatric diversification?**

Our main finding, that morphology is closely linked to ecological resource use in this isolated population of Midas cichlids, indicates that individuals exploit habitat according to their morphology. In this regard, it is conceivable that substantially different phenotypes are favored in both habitats, somewhat underlining the role of disruptive selection in shaping this population, as discussed in (Schluter & McPhail, 1992). Ecological speciation theory predicts bimodal trait distribution in ecologically-relevant traits when divergent selection is at work in progressing towards sympatric speciation (Bolnick, 2011; Nosil, 2012; Schluter, 2000). Despite of the observed large variance in a number of ecologically-relevant traits, we found rather continuous distributions in the eco-morphological data sets we investigated (Table 2). Similarly, when all variables were jointly considered, a single cluster was statistically most likely. This rejects the possibility of discrete eco-morphs (Elmer et al., 2010a) and suggests that *A. tolteca* is a solitary (endemic) Midas cichlid species confined to Asososca Managua.

However, there were some variables that were not simply best explained by a single continuous and normal distribution (Table 2, Figure S7). Platykurtosis was nearly significant in LPJ width. Further, the null-hypothesis of normality was close to being rejected in a number of other data sets such as $\delta^{15}$N and BHI. Altogether, this suggests at most a moderate discontinuous distribution for these traits, which may be indicative and interpreted as signatures of incipient divergent ecological selection. Whether these discontinuities would become more pronounced over time and whether reproductive isolation will eventually be achieved based on this, remain open questions. But, given the extremely young age of this lake it might be surprising to find so much phenotypic diversity and ecological specialization at all.

We did not find any neutral genetic sub-structuring in our sample of *A. tolteca*
specimens that might indicate signatures of assortative mating by ecotype suggesting no reproductive isolation within the lake. Indeed, genetic diversity in Asososca Managua is low (0.549) compared to other crater lakes that are known to house multiple described species (Apoyo: 0.590 (Barluenga & Meyer, 2010), Xiloá: 0.668 (Barluenga & Meyer, 2010)), but that again, are also at least five to ca. twenty times older.

In summary, the broad and mostly continuous ecological variation within Asososca Managua cichlids, in the absence of detectable genetic differentiation, might thus facilitate and/or correspond to an early stage of incipient sympatric ecological speciation (Hendry et al., 2009).

**Convergent benthic-limnetic divergence**

It has been hypothesized that adaptive radiation and speciation (in cichlids) proceed by following a particular scheme (Gavrilets & Losos, 2009; Kocher, 2004; Streelman & Danley, 2003): in the first place a niche-use divergence into macrohabitats, followed by further eco-morphological divergence and finally differentiation based on traits relevant for communication and genetic differences. Speciation is often seen as a continuous process with several intermediate stages differing in the degree of adaptive ecological variation and reproductive isolation (Hendry et al., 2009).

Given that the Asososca Managua Midas cichlid population is youngest among those living in several other Nicaraguan crater lakes that have experienced speciation along the benthic-limnetic axis, it is of particularly interest and relevance for the study of speciation. The Midas cichlid population of Asososca Managua exhibits the most recent colonization history (maximally 1,245 years (Pardo et al., 2008)), followed by lakes Xiloá with ca. 5,400 years (Elmer et al., 2013) and Apoyo being the oldest, with ca. 20,000 years (Barluenga et al., 2006). We assume that these crater lakes initially did not substantially differ in the nature of environmental conditions. Indeed, the convergent evolution of multiple independently evolved sympatric Midas cichlid species communities (Apoyo (Barlow & Munsey, 1976; Elmer et al., 2010a; Geiger et al., 2010; Stauffer Jr et al., 2008) and Xiloá (Elmer et al., 2010a; Recknagel et al., 2013; Stauffer Jr & McKaye, 2002)) and phenotypes (Figure 17, Figure 18A) lends indirect support that different crater lakes are indeed comparable and raises the interesting option that the evolutionary fates in these crater lakes might be predictable, as has been
assumed for other systems (Morris, 2006; Morris, 2010; Robinson & Wilson, 1994). However, the strength of selection in promoting diversification along the benthic-limnetic axis may vary across lakes. We have recently analyzed some abiotic features of the crater lakes of Nicaragua in this regard and found that the depth and availability of benthic environments are the best predictors of the degree of body elongation of the respective endemic Midas cichlid species (Recknagel et al., in prep.). Further, in the crater lake scenario, the number and population densities of other competing species along with their colonization times must have impacted the Midas cichlid radiations in lakes Apoyo and Xiloá.

Asososca Managua has a bathymetric profile that is extraordinarily biased towards the open water habitat (Recknagel et al., 2013). Therefore, and given the characteristic body shape, *A. tolteca* is notably considered a limnetic species that is variable along the benthic-limnetic axis. The absence or secondary loss of a benthic-specialised species is consistent with theoretical modelling of Midas cichlid speciation in volcanic crater lakes (Gavrilets et al., 2007). It is conceivable that the present Asososca Managua Midas cichlid population (*A. tolteca*) (mostly limnetic, but also consisting of many individuals that do not conform to the common type) constitutes an intermediate stage of speciation along the benthic-limnetic axis, especially when divergent selection is weak (Gavrilets et al., 2007). Indeed, with intermediate strengths of selection, speciation along the benthic-limnetic axis can be achieved rapidly from a benthic ancestral species (Gavrilets et al., 2007), a theoretical scenario that has been underpinned by a recent empirical study (Kautt et al., 2012) for lakes Apoyo and Xiloá.

Many ecological and evolutionary parameters regarding the Midas cichlids system are still largely unknown, and their clarification would mark substantial progress in the understanding and modelling of diversification in these lakes (Gavrilets et al., 2007). These include the clarification of the underlying genetic architecture of ecology and mate choice related traits as well as estimates of population density and individual dispersal, although some progresses have been made to address these issues (e.g. (Dittmann et al., 2012; Fruciano et al., in prep; Machado-Schiaffino et al., in prep.).
Nonetheless in light of the recognized parallel evolution along the benthic-limnetic axis, we conclude that Midas cichlids in this small crater lake are likely about to further diversify along this axis, making them a candidate for investigating the process of ongoing lineage diversification and its relevant phenotypic components.

Conclusions
Convergent evolution and speciation of teleost fishes along the benthic-limnetic axis has been extensively documented. The present study focuses on the young adaptive radiation of Nicaraguan crater lake Midas cichlids that also has experienced convergent evolution along the benthic-limnetic axis. In *A. tolteca*, the youngest of the crater lake species in the species complex, we demonstrated high degrees of individual variation in phenotypic and diet traits that are known to be main targets of divergent selection along the benthic-limnetic axis. We also found a robust pattern of among-individual variation in ecological resource use (individual specialization). The observed pattern is likely to reflect disruptive selection and the involvement of frequency-dependent processes resulting from intraspecific competition and might be essential for future intralacustrine divergence. *A. tolteca* explores a relatively wider ecological space than the older and more species rich Midas cichlid communities in other crater lakes that have completed speciation events along the benthic-limnetic axis, possibly indicating ongoing character release following ecological opportunity. Given the rapid and independent advent of two other limnetic Midas cichlid species from likely generalist ancestors, we expected, following adaptive radiation theory, to also detect signatures of divergent selection in this young crater lake species. At the present stage, all investigated traits were rather explained by continuous than discrete or bimodal trait distributions and there was no genetic structuring. Hence any attempt to assign *A. tolteca* into discrete ecotypes, or even species is not supported by these data. In a larger context of research on adaptive radiations, among the Midas cichlid radiations, that of Asososca Managua is particularly interesting since it is the youngest of them all where incipient speciation is ongoing and diversifying responses to disruptive selective forces still developing.
Acknowledgements

We thank Ministerio del Ambiente y los Recursos Naturales (MARENA) for sample collection permits. We are grateful to Empresa Nicaragüense de Acueductos y Alcantarillados (ENACAL), particularly Martin Brenes for generously supporting this study. Thanks to Maria-Luise Spreitzer, Carmelo Fruciano and Gonzalo Machado-Schiaffino for their help with the sampling. We kindly thank Jonathan B. Losos for his helpful comments on this manuscript. Thanks to Julian Torres-Dowdall for fruitful discussions. We thank Sebastian Stockmaier for his help with the jaw extraction. Thanks to the Stable Isotope Group of the Limnological Institute at the University of Konstanz. Special thanks to Dr. Fränzi Korner-Nievergelt for her advise with statistical analysis. Funding was provided through various grants of the Deutsche Forschungsgemeinschaft to AM, an Alexander von Humboldt fellowship to KRE, a European Research Council (ERC) advanced grant 293700 “GenAdap” to AM and of Deutsche Cichliden Gesellscha to HK.
Chapter IV

Mouth asymmetry in the textbook example of scale-eating cichlid fish is not a discrete dimorphism after all

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Published in *Proceeding of the Royal Society (B)*: 279 (1748): 4715-23 (2012)

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Abstract
Individuals of the scale-eating cichlid fish, *Perissodus microlepis*, from Lake Tanganyika tend to have remarkably asymmetric heads that are either left-bending or right-bending. The “left” morph opens its mouth markedly towards the left and preferentially feeds on the scales from the right side of its victim fish and the “right” morph bites scales from the victims’ left side. This striking dimorphism made these fish a textbook example of their astonishing degree of ecological specialization and as one of the few known incidences of negative frequency-dependent selection acting on an asymmetric morphological trait, where left and right forms are equally frequent within a species. We investigated the degree and the shape of the frequency distribution of head asymmetry in *P. microlepis* to test whether the variation conforms to a discrete dimorphism, as generally assumed. In both adult and juvenile fish, mouth asymmetry appeared to be continuously and unimodally distributed with no clear evidence for a discrete dimorphism. Mixture analyses did not reveal evidence of a discrete or even strong dimorphism. These results raise doubts about previous claims, as reported in textbooks, that head variation in *P. microlepis* represents a discrete dimorphism of left- and right-bending forms. Based on extensive field sampling that excluded ambiguous (i.e. symmetric or weakly asymmetric) individual adults, we found that left and right morphs occur in equal abundance in five populations. Moreover, mate pairing for 51 wild caught pairs was random with regard to head laterality, calling into question reports that this laterality is maintained through disassortative mating.

Introduction
Morphological variation plays an important role in the ecology and evolution of natural populations. One particularly interesting case of phenotypic variation is “left-right asymmetry”, where phenotypic traits of left or right individuals differ asymmetrically (Palmer, 2004). Left-right asymmetry can be found in several phyla of animals, for example, in eye side in flatfishes (Pleuronectiformes) (Hubbs & Hubbs, 1945), direction of shell coiling in the snails, *Euhadra* species (Davison et al., 2005), the major claws of the male mud shrimp, *Neotrypaea californiensis* (Labadie & Palmer, 1996) and direction of the mouth-opening in the scale-eating cichlid fish, *Perissodus microlepis*
The adaptive radiations of cichlid fishes in the great East African lakes, including Lakes Malawi, Tanganyika and Victoria, consist of hundreds of endemic species each (Fryer & Iles, 1972). The co-existence of so many species demands astonishingly precise ecological specializations of these species, to avoid competitive exclusion. *Perissodus microlepis*, a scale eating cichlid fish from Lake Tanganyika, is a textbook example of the evolution of a pronounced morphological asymmetry and of a striking trophic specialization (Hori, 1993). Most of the nine described species of cichlids of the monophyletic tribe Perissodini from Lake Tanganyika (Koblmüller et al., 2007), including *P. microlepis*, are specialized to feed predominantly on the scales of other fish (Fryer, 1955; Fryer & Iles, 1972; Marlier & Leleup, 1954). In *P. microlepis*, individuals are even more specialized: in individual fish the mouth bends either to the left (“left” morph; L) or to the right (“right” morph; R) (Figure 19).

![left morph (L) right morph (R)](image)

**Figure 19.** Two morphologically pronounced morphs of the scale eating cichlid, *Perissodus microlepis*. The left individual is a clear L-morph with the mouth bend to the left. The individual on the right side has the mouth bend towards the right (R-morph). L-morphs preferentially attack the right flank of their prey while R-morphs attack their
victim’s left flank (Hori, 1993; Lee et al., 2010; Takeuchi et al., 2012; Van Dooren et al., 2010). Some textbooks in evolutionary biology hence present the asymmetrical mouth of *P. microlepis* as a typical example of antisymmetry (AS), where two distinct forms co-exist (i.e. dimorphic asymmetry) (e.g. (Futuyma, 2009)).

Mouth laterality in *P. microlepis* is thought to facilitate efficient scale eating because the contact area between the predator’s teeth and the flank of the prey fish is enlarged (Hori, 1993). The frequency of L- and R-morphs in natural populations of *P. microlepis* was found to fluctuate about an approximately 50/50 ratio over more than a decade of sampling (Hori, 1993) and negative frequency-dependent selection has been invoked to explain the relative stability of this 50/50 ratio (Hori, 1993). The more common morph is thought to suffer a fitness disadvantage since prey fish would be more often attacked from the preferred side, making them more alert to attacks from that direction. Therefore, the more common morph of the predator would be expected to succeed less often in removing scales than the less-common morph, which in turn would be favoured (Hori, 1993).

Although *P. microlepis* is well-known among evolutionary biologists, many fundamental questions about this fish remained unanswered (Palmer, 2010). Contradictory hypotheses have been proposed about the trait distribution and the inheritance of mouth asymmetry in this fish. Differences in the mouth asymmetry between L- and R-morphs of adult as well as juvenile fish are sufficiently pronounced to be discernable upon visual inspection by trained observers (Hori, 1993; Hori et al., 2007). A strictly bimodal distribution (“strong” AS) (Palmer & Strobeck, 1992) of the asymmetry has been previously reported for this species (Hori, 1993; Hori et al., 2007). However, the bimodality of this asymmetry has been called into question due to recent reports of unimodally distributed mouth asymmetry at the late larval stage (Stewart & Albertson, 2010) as well as from adult *P. microlepis* (Van Dooren et al., 2010). Further, mouth asymmetry in adults of this species was found to conform to “weak” AS (Van Dooren et al., 2010) – where the trait distribution is rather platykurtic than strictly bimodal (Palmer & Strobeck, 1992). Yet the degree and distribution of mouth asymmetry in juvenile fish has not been investigated so far.
Moreover, the degree of genetic determination of this remarkable asymmetry remains unknown. Previously, two alleles at a single Mendelian locus were thought to determine the directionality of mouth asymmetry (Hori, 1993; Hori et al., 2007; Stewart & Albertson, 2010). However, a recent review (Palmer, 2010) pointed out that the observed phenotype frequencies among offspring of breeding pairs of different morph combinations were inconsistent with models of Mendelian inheritance. Additionally, reported intra-specific brood mixing (Yanagisawa, 1985) could affect phenotype frequencies within broods collected in the field, which would make interpretation of field data, particularly without genetic knowledge on parentage, difficult.

Disassortative mating – where individual *P. microlepis* fish preferentially mate with individuals of the opposite morph – was thought to be an additional mechanism in stabilizing the variation in morph frequency over time (Takahashi & Hori, 2008) because offspring frequencies exhibited a 1:1 ratio of L- to R-individuals for disassortatively breeding pairs, a 1:2 ratio for RR pairs and a 1:0 ratio for LL pairs. These offspring frequencies were explained by a complex inheritance pattern involving two alleles at a single locus where R is dominant over L and R is homozygous lethal (Hori et al., 2007; Takahashi & Hori, 2008). However, phenotypically plastic responses to handed foraging behavior have recently been suggested to give rise to these mouth asymmetries (Van Dooren et al., 2010). Furthermore, because no genetic basis for laterality has been found in most cases of AS (Palmer, 2004; Palmer, 2009), phenotypic plasticity may be an important source of phenotypic variation in this system.

The suggested disassortative mating pattern in *P. microlepis* is difficult to reconcile with two recent findings: (1) phenotypic plasticity apparently affects mouth asymmetry (Van Dooren et al., 2010) and (2) genetic evidence suggests that random mating occurs in natural populations (Lee et al., 2010). The sample size of pairs of *P. microlepis* caught in the field so far has been rather small (*n* = 24) (Takahashi & Hori, 2008) and a reasonable explanation for how mating partners should recognize each others’ laterality was lacking.

Here, we address these open questions surrounding mouth laterality of this species by revisiting the mating pattern and population-based relative abundances of mouth morphs and by assessing the (multivariate) phenotypic trait distributions of adults and juveniles. In particular, we test whether mouth asymmetry of *P. microlepis*
displays dimorphic (antisymmetric) variation, either strong or weak, as has been so far believed.

**Material and Methods**

(a) **Sample collection, and assessments of equal abundance and mating pattern**

We sampled 287 adult individuals of *Perissodus microlepis* from five different locations around M pulpungu (Zambia) on the southern tip of Lake Tanganyika in April 2010 from water depths up to 7 m (Table 3). Most specimens were caught by gill nets and sampled randomly with respect to mouth opening direction. All fish were photographed live from a lateral view and then preserved in 97 % ethanol and vouchered in the fish collection at the University of Konstanz. Standard length (SL) was determined from digital photographs.

In addition, sixty-five juveniles from five different broods (of three RR pairs and two RL pairs) were caught and transported to the animal care facility at the University of Konstanz. The broods were raised in separate 40 l and later 200 l aquaria on *Artemia* nauplii and flake food as diet. These juvenile *P. microlepis* were used for quantitative measures of mouth asymmetry as well as for laboratory feeding experiments (Lee et al., 2012 - Chapter V of the present thesis). Due to the observed surprisingly high levels of brood-mixing (Lee et al., unpublished data), we could not reliably investigate inheritance patterns of mouth asymmetry and therefore only focus on trait distributions.

Assessments of morph abundance and mating pattern were based upon visual determination of mouth laterality in the field. Laterality of 287 live adult fish was judged by eye by two independent observers (HK and AM). “Mouth opening direction” was the criterion for laterality (i.e. the relative orientation of the ascending process of the premaxillary bone, while the mouth is opening and the jaw protruding). Ambiguous specimens (*n* = 23), defined as those individuals where the two observers made no or different calls (i.e. symmetric or weakly asymmetric individuals), were excluded from the Chi-square (*χ*²) analyses to test whether the ratio of L- and R-morphs deviated from equal abundance.

To assess the mating pattern in *P. microlepis*, 54 breeding pairs were collected by diving with hand nets at Toby Veall’s Lodge (S 08°37.4’ E 031°12’), about 20 km
north-east of Mpulungu at the southern tip of Lake Tanganyika. Three of the 54 pairs were excluded from the analysis because one breeding partner was found to be ambiguous with respect to laterality by the two observers ($n = 2$) or scored with opposite scores ($n = 1$). Expected numbers of RR, RL and LL pairs were calculated from the laterality frequencies in our sample and compared to observed frequencies with $\chi^2$–statistics.

(b) Quantifications of mouth asymmetry in adult $P. \text{microlepis}$

We used statistical tools developed for fluctuating asymmetry (FA) analyses (Palmer & Strobeck, 2003) to test whether mouth asymmetry in $Perissodus \text{microlepis}$ corresponded to dimorphic variation (e.g. antisymmetry), even though we recognize that the jaw asymmetry variation in this species may have predictable genetic or environmental causes (e.g. (Van Dooren et al., 2010)), i.e. it may not be due solely to developmental noise, as is normally assumed for FA variation.

Mouth asymmetry in adult fish was quantified by estimating mouth bending angle (Hori et al., 2007), “$\alpha L - \beta R$” (in °) for 249 preserved individuals from standardized photographs from a dorsal view. A randomly selected sub-sample ($n = 33$) was photographed twice independently, and replicate and blind measurements were done from the repeated photographs to assess measurement error (ME) caused by photographing. Potential ME caused by preservation could not be assessed. ME1 – average difference between pairs of measurements on each L and R side ($\sum |M_1 - M_2|/n$) (Palmer & Strobeck, 2003) – was calculated. ME3 – ME as % of non-directional asymmetry (FA and AS) – was further estimated from results of a two-way mixed model ANOVA ($100 \times \text{MS}_{\text{error}}/\text{MS}_{\text{sides} \times \text{individuals}}$) ((Palmer & Strobeck, 2003); see below).

Mouth angles were measured in tpsDig v. 2.16. (Rohlf, 2010a). On each image, a triangle connecting the anterior-most points of the eye sockets and the tip of the snout was drawn to calculate angles, $\alpha L$ (angle of the vertex by the left eye) and $\beta R$ (angle of the vertex by the right eye; see below). Negative values of $\alpha L - \beta R$ indicate right-bending specimens, whereas positive values indicate left-bending individuals. The most extreme fish (mouth bending angles deviating by more than 15°; $n = 11$) were excluded from analyses because these fish or parts of them seemed to have been deformed during
A detailed analysis of mouth bending angle was performed in R (R Core Team, 2012) to test whether mouth asymmetry in *P. microlepis* represented dimorphic variation [e.g. antisymmetry (AS); a bimodal or platykurtic distribution], directional asymmetry (DA; a significant departure of mean asymmetry from zero) or fluctuating asymmetry (FA; a continuous normal distribution of mean zero). First, the trait mean for the whole sample as well as for each separate population (with large sample sizes of \( n = \text{ca. 50} \)) was calculated and analyzed for a departure of the mean from zero to test for DA (Palmer, 1994). As suggested in (Van Dooren et al., 2010), the dip statistic (Hartigan & Hartigan, 1985), a specific test for a unimodal distribution was used to evaluate any presence of a “strong” AS. One would expect a bimodal distribution if AS were strong (Palmer & Strobeck, 1992). Linear regression analyses were also conducted to test for a significant relationship between degree of mouth asymmetry and body size (Hori et al., 2007).

Using repeated measurements (angles) from replicate images of the sub-sample that was used for the assessment of ME, a two-way mixed model ANOVA (sides = fixed; individuals = random) was carried out to test if mouth asymmetry exhibits DA or non-directional asymmetry as suggested by other studies (Merilä & Björklund, 1995; Palmer & Strobeck, 1986). This analysis tests for the significance of directional (effects of “sides”) and non-directional asymmetries (effects of “sides \( \times \) individuals”) relative to the variation expected due to ME (Palmer & Strobeck, 1986).

To further test whether the observed trait distribution consisted of more than a one-component normal distribution, a mixture analysis was performed. An Anscombe-Glynn test for platykurtosis (Anscombe & Glynn, 1983) in the trait distribution was used to evaluate the presence of a potential “weak” AS following (Van Dooren et al., 2010). A broad-peaked, but still unimodal (i.e. platykurtic) distribution would be expected if AS were weak (Palmer & Strobeck, 1992). Further, using an expectation–maximization (EM) algorithm-based approach (McLachlan & Peel, 2000) implemented in the mixtools package (Benaglia et al., 2009), mixtures of two single-component normal distributions were fitted to the mouth asymmetry data and their fit was inspected (Figure S8). A parametric bootstrap test (1000 iterations) using the output of the EM mixture model fits was performed to test whether the trait distributions consisted of one
or two components (Benaglia et al., 2009).

(c) Quantifications of mouth asymmetry in juvenile *P. microlepis*

For the live juvenile *P. microlepis*, we employed two different methods to quantify mouth asymmetry from photographs: (1) mouth bending angle (Hori et al., 2007) and (2) geometric morphometric analysis based on mouth landmarks. Each live test fish was photographed from a dorsal view in a standardized position using a Zeiss Axiophot digital microscope (Zeiss, Germany). Mouth bending angle was measured at two ontogenetic stages, of ca. three months (\(n = 65\)) and ca. seven months (\(n = 54\)) of age. A sub-sample of 24 individuals from each of the two age groups was photographed twice independently to quantify ME, as was performed in adults (see above). Statistical analyses were conducted as done in adults.

We also performed geometric morphometric analyses on landmark configurations for the combined age groups. A set of ten landmarks defining mouth shape was digitized using tpsDig v. 2.16 (Rohlf, 2010a), consisting of two un-paired landmarks (snout tip; mid-point between the frontal eye sockets) as well as four paired landmarks (see Figure S9 for detailed description).

Using MorphoJ 1.02e (Klingenberg, 2011), we applied a generalized Procrustes superimposition taking object symmetry into account (Klingenberg et al., 1998; Klingenberg et al., 2002; Mardia et al., 2000). This method compares the original mouth shapes to their respective mirror-images and partitions total variation in mouth shape into components of symmetry as well as of asymmetry (Klingenberg et al., 2002). Symmetric components represent variation in the left-right averages of mouth shape whereas asymmetric components indicate the left-right mouth asymmetries within individuals (Klingenberg et al., 2002). Therefore, we only considered the asymmetric components of variation in mouth shape.

Principal component analysis (PCA) was used to identify the major patterns of shape change in the asymmetric component of shape variation that relate to mouth orientation. Individual loadings on the most important asymmetric component affecting mouth asymmetry (PC-1) were extracted and their distribution was analyzed in the same way as the mouth bending angle data (see above). Moreover, discriminant function analysis (DFA) was performed between both age groups (three- vs. seven-month old
fish) to test for effects of age (body size) on the mouth asymmetry means. DFA is a commonly used ordination-based multivariate method to examine whether a priori determined two groups can be distinguished reliably (Albrecht, 1980; Mardia et al., 1979; Timm, 2002; Zelditch et al., 2012). Levene’s test (Levene, 1960) was further conducted using individual PC-1 scores to investigate whether variance in mouth asymmetry differed significantly between both age groups.

To statistically test for the magnitude of DA and non-directional asymmetry relative to ME, we landmarked a sub-sample of 24 individuals twice on replicate images and further conducted Procrustes analysis of variance (ANOVA) as well as multivariate analysis of variance (MANOVA) on each age group (Klingenberg et al., 2002; Mardia et al., 2000). For the latter analysis, only the asymmetric component of shape variation was considered. The Procrustes ANOVA procedure considers fewer parameters than the MANOVA model, but assumes equal amount and direction of variation in each landmark (Klingenberg et al., 2002), whereas the MANOVA procedure avoids such assumptions (Mardia et al., 2000). These statistical analyses have been commonly applied in studies of multivariate shape analyses in relation to left-right asymmetry (Klingenberg & McIntyre, 1998; Klingenberg et al., 2002; Klingenberg, 2003; Mikula & Macholán, 2008).

The morphological data (e.g. mouth bending angle, body size) and morphometric data have been deposited on DRYAD entry doi:10.5061/dryad.4605c.
Results

(a) Equal abundance of mouth morphs and random mating in *P. microlepis*

The direction of mouth laterality could be unambiguously assigned for 92% (264 of 287 individuals) of the field-collected adult *P. microlepis* by visual inspection (table 1). Only 18 individuals (6%) were independently scored as no calls (ambiguous) and five individuals (2%) were scored differently by both experimenters (HK and AM). The average fish standard length (SL) was 7.95 cm (range: 5.52 – 11.13 cm; SD = 1.21 cm). None of the five sampling locations showed deviations from an equal ratio of R- to L-morphs (Table 3) corroborating the earlier hypothesis of equal abundance (Hori, 1993; Lee et al., 2010).

Table 3. Abundance of lateral morphs in *Perissodus microlepis* from five different populations.
Sample sizes -- relative frequencies in parentheses. Bold numbers indicate the number of specimens that could not be unambiguously assigned.

<table>
<thead>
<tr>
<th>Sample Location</th>
<th>Right Morph</th>
<th>Left Morph</th>
<th>Total</th>
<th>$\chi^2$</th>
<th>$p$ - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crocodile Island</td>
<td>2 -- (50%)</td>
<td>2 -- (50%)</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kasakalawe Village (S 08°46.8' E 031°04.8')</td>
<td>22 -- (43%)</td>
<td>29 -- (57%)</td>
<td>51 + 3</td>
<td>0.7</td>
<td>0.403</td>
</tr>
<tr>
<td>Katoto Village (S 08°48.2' E 031°1.3')</td>
<td>33 -- (56%)</td>
<td>26 -- (44%)</td>
<td>59 + 3</td>
<td>0.6</td>
<td>0.431</td>
</tr>
<tr>
<td>Mbita Island (S 08°45.3' E 031°05.4')</td>
<td>21 -- (47%)</td>
<td>24 -- (53%)</td>
<td>45 + 13</td>
<td>0.1</td>
<td>0.777</td>
</tr>
<tr>
<td>Toby Veall's Lodge (S 08°37.4' E 031°12')</td>
<td>57 -- (54%)</td>
<td>48 -- (46%)</td>
<td>105 + 4</td>
<td>0.6</td>
<td>0.439</td>
</tr>
<tr>
<td>all locations pooled</td>
<td>135 -- (51%)</td>
<td>129 -- (49%)</td>
<td>264 + 23</td>
<td>0.1</td>
<td>0.752</td>
</tr>
</tbody>
</table>

The specimens used for our investigation of mating patterns consisted of 55 R- and 47 L-morphs that formed 51 pairs. Thirteen pairs were RR, 9 were LL and 29 were RL. Although RL pairs were proportionally more common than expected, the observed pair frequencies were not significantly different from expected frequencies under the assumption of random mating ($\chi^2 = 1.06$, df = 2; $p = 0.59$; Figure 20).
(b) Mouth asymmetry in adult *P. microlepis*

ME1 of angles $\alpha_L$ and $\beta_R$ was 1.71° and 1.91° respectively, which accounts for 11.3% of non-directional asymmetric (FA and AS) variation (ME3), a finding similar to that of other studies reported in (Palmer & Strobeck, 1986). Note that when referring to this variation as FA, we do so only in the sense of the shape of the frequency distribution of mouth asymmetry variation; we do not mean to imply that the primary cause of this variation is developmental noise, as is generally assumed in studies of FA variation.

The outcome of our detailed analysis of mouth bending angle of 238 specimens revealed that mouth asymmetry did not significantly depart from unimodality in any of the populations or in the pooled sample (Table 4, Figure 21), ruling out the possibility of strong AS. The trait mean was always found not to be significantly different from zero (Table 4), indicating no DA. The two-way mixed model ANOVA confirmed the initial indication of no DA in our sample whereas it revealed highly significant non-directional asymmetry relative to ME (Table 5).

The Anscombe-Glynn tests revealed no significant platykurtosis in the asymmetry distribution and the mixture analyses along with the parametric bootstrap test strongly suggest that a single-component normal distribution best fitted our sample, both as a whole and at the population level (Table 4, Figure S8). However, in the pooled
sample platykurtosis was only marginally non-significant \( (p = 0.06) \), which does not allow us to exclude the possibility of weak AS.

Further, no significant correlation was detected between the size of the fish and mouth bending angle except in one population (Katoto Village: Table 4, Figure 21), which suggests that mouth asymmetry does not necessarily increase with body size as hypothesized by (Hori et al., 2007). However, in the pooled sample the effect was on the borderline of being significant \( (p = 0.07; \text{Table } 4) \), so this association cannot be rejected with confidence.

**Figure 21. Trait distribution of mouth asymmetry in adult *Perissodus microlepis* from four wild populations.**

Left panel: pooled sample. Four right panels: analyses at the population level. Top: histograms of the distribution of mouth bending angle in the respective samples. Middle: smoothed histograms to exclude potential effects that might be caused by the number of bins. Bottom: linear regression between standard body length and degree of mouth asymmetry.
Table 4. Statistical analyses of traits related to mouth asymmetry in adult and juvenile *Perissodus microlepis*.

These analyses include the dip tests for unimodality, one sample $t$-tests for the assessment of the deviation of the trait mean from zero, Anscombe-Glynn tests for platykurtosis, mixture analyses to determine the number of components and linear regression analyses to test for a significant correlation between mouth bending angle and body size. $p$-values in boldface denote statistical significance. (a) Mouth bending angle of wild-caught adult specimens (whole sample and listed by population). (b) Mouth bending angles of laboratory-reared juvenile specimens. (c) Individual PC-1 scores, derived from a PCA on the asymmetric component of shape variation.

<table>
<thead>
<tr>
<th></th>
<th>dip test</th>
<th>one sample t-test</th>
<th>platykurtosis</th>
<th>mixture analysis</th>
<th>linear regression analysis</th>
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<tr>
<td></td>
<td>$n$</td>
<td>trait mean</td>
<td>dip</td>
<td>$t$</td>
<td>$p$</td>
</tr>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toby's Lodge</td>
<td>77</td>
<td>-0.53 [°]</td>
<td>0.028</td>
<td>&gt; 0.5</td>
<td>-0.69</td>
</tr>
<tr>
<td>Karakalave</td>
<td>43</td>
<td>-1.13 [°]</td>
<td>0.046</td>
<td>&gt; 0.5</td>
<td>-1.24</td>
</tr>
<tr>
<td>Katoto</td>
<td>58</td>
<td>-1.57 [°]</td>
<td>0.036</td>
<td>&gt; 0.1</td>
<td>-1.85</td>
</tr>
<tr>
<td>Mbita Island</td>
<td>32</td>
<td>0.62 [°]</td>
<td>0.046</td>
<td>&gt; 0.1</td>
<td>0.86</td>
</tr>
<tr>
<td>all samples</td>
<td>238</td>
<td>-0.58 [°]</td>
<td>0.015</td>
<td>&gt; 0.5</td>
<td>-1.46</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ca. 3 months</td>
<td>65</td>
<td>0.58 [°]</td>
<td>0.029</td>
<td>&gt; 0.5</td>
<td>1.84</td>
</tr>
<tr>
<td>ca. 7 months</td>
<td>54</td>
<td>0.64 [°]</td>
<td>0.047</td>
<td>&gt; 0.1</td>
<td>1.95</td>
</tr>
<tr>
<td>(c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ca. 3 months</td>
<td>65</td>
<td>3.31x10^-5</td>
<td>0.037</td>
<td>&gt; 0.5</td>
<td>0.03</td>
</tr>
<tr>
<td>ca. 7 months</td>
<td>54</td>
<td>-3.99x10^-5</td>
<td>0.036</td>
<td>&gt; 0.5</td>
<td>-0.02</td>
</tr>
</tbody>
</table>
Table 5. Statistical tests for the significance of directional asymmetry and non-directional asymmetry

Two-way mixed-model ANOVA (sides = fixed, individuals = random) was used, following (Palmer & Strobeck, 1986), to test whether the trait distributions are rather concordant with directional asymmetry (“sides”) or with non-directional asymmetry (“sides × individuals”; fluctuating asymmetry and antisymmetry) relative to measurement error (imaging error). *P*-values in boldface denote statistical significance.

<table>
<thead>
<tr>
<th>effect</th>
<th>MS</th>
<th>df</th>
<th><em>F</em></th>
<th><em>p</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>adults</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sides</td>
<td>16.614</td>
<td>1</td>
<td>0.65</td>
<td>0.426</td>
</tr>
<tr>
<td>individuals</td>
<td>25.222</td>
<td>32</td>
<td>0.986</td>
<td>0.516</td>
</tr>
<tr>
<td>sides × individuals</td>
<td>25.579</td>
<td>32</td>
<td>8.823</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>measurement error</td>
<td>2.899</td>
<td>66</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ca. three months</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sides</td>
<td>13.104</td>
<td>1</td>
<td>2.394</td>
<td>0.135</td>
</tr>
<tr>
<td>individuals</td>
<td>4.545</td>
<td>23</td>
<td>0.831</td>
<td>0.670</td>
</tr>
<tr>
<td>sides × individuals</td>
<td>5.473</td>
<td>23</td>
<td>7.706</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>measurement error</td>
<td>0.710</td>
<td>48</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ca. seven months</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sides</td>
<td>14.420</td>
<td>1</td>
<td>2.344</td>
<td>0.139</td>
</tr>
<tr>
<td>individuals</td>
<td>3.088</td>
<td>23</td>
<td>0.502</td>
<td>0.947</td>
</tr>
<tr>
<td>sides × individuals</td>
<td>6.151</td>
<td>23</td>
<td>17.354</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>measurement error</td>
<td>0.354</td>
<td>48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(c) *Mouth asymmetry in juvenile P. microlepis*

The ME1 of angles in the three month old juvenile fish was 0.93° (α L) and 1.02° (β R) while the ME1 of the seven month old juvenile fish was smaller, 0.58° (α L) and 0.76° (β R). This amount of ME accounts for 13.0 % and 5.8 % of the non-directional asymmetric variation (ME3) for the three and seven month old fish, respectively.

The degree of mouth asymmetry in juvenile fish was considerably lower than in wild-caught adults and showed a continuous and unimodal distribution across the two different ontogenetic stages (Table 4, Figure 22). These data reject the hypothesis of strong AS.
Twenty-one of the 65 three month old fish (32 %) displayed nearly symmetric mouth morphologies (i.e. mouth bending angles ranging between − 1° and + 1°; Figure 22 A-C) as did 18 out of 54 individuals (33 %) measured at seven months of age. The average mouth bending angle was 0.58° and 0.64° for the three and seven month old fish, respectively and was not significantly different from zero despite being close to statistical significance (Table 4). The two-way mixed ANOVA of each age group provided no support for DA, but a highly significant non-directional asymmetry relative to ME was detected (Table 5). No sign of platykurtosis was detected and a single-component normal distribution best fitted the samples of both age groups (Table 4, Figure S8). Also in the seven month old lab-reared fish the laterality did not increase significantly with body size (Table 4).

Similar to the outcome of the analysis of mouth bending angles, individual PC-1 scores also exhibited a continuous and unimodal distribution (Table 4; Figure 22D-F). However, the three month old fish showed significant platykurtosis in the distribution of PC-1 scores. Nevertheless, the distribution most likely consisted of only one-component normal distribution (Table 4, Figure S8). PC-1 accounted for 67.22 % of the total variation in asymmetric mouth shape components. The shape change along PC-1 was clearly associated with the expected pattern of mouth asymmetry (Figure 22G). The DFA on the asymmetric component of shape variation showed that mouth shape means of both age groups were not significantly different (Procrustes distance = 0.0023; \( p = 0.63 \)). However, shape variance (as inferred from PC-1) was significantly higher in the seven month old fish than in the three month old fish (Levene’s test; \( F = 11.625; \ p = 0.001 \), Figure 22H).

The multivariate analysis of mouth orientation also showed that the trait distributions in both age groups fitted better to non-directional asymmetry than to DA. The Procrustes ANOVA of geometric morphometric data showed that non-directional asymmetry (sides × individuals) was statistically highly supported for both age groups, although DA (sides) was also slightly significant only for the three month old group (Table S6). Considering the MANOVA (nonisotropic model), both DA and non-directional asymmetry were statistically highly significant in the three month old fish, but not in the seven month old fish (Table S6).
Figure 22. Frequency distribution of traits related to mouth asymmetry in lab-reared juvenile *Perissodus microlepis*.

A: The triangle indicates connection lines between the frontal points of the two eye sockets and the upper-jaw intersection, drawn to calculate angle differences between αL (angle of the vertex by the left eye) and βR (angle of the vertex by the right eye) on a juvenile *P. microlepis* (Hori et al., 2007). Histograms and density plots of mouth bending angle distributions of the three month (B) and seven month old fish (C). D: A juvenile *P. microlepis* individual displaying the landmark set that was used for geometric morphometric analyses (see Figure S9 for detailed description of how the landmarks were defined). Histograms and density plots of individual PC1-scores for the three month (E) and seven month old (F) fish cohorts. G: Shape changes that are associated with PC1. Lines drawn in light blue represent an average consensus mouth shape, whereas dark blue lines indicate the shift in mouth shape that is associated with 0.1 units in the positive direction on PC1 in (H). H: The plot of PC1 vs. PC2 displays the difference in variance along PC1 between both age groups. Individual scores and 95% confidence intervals indicated in blue and red for three and seven months old fish, respectively.
Discussion

The pronounced laterality of the foraging behavior and asymmetry in the morphology of \textit{P. microlepis} has made this species a textbook example (Futuyma, 2009) of both their astonishing degree of adaptation and specialization and of negative frequency-dependent selection (Hori, 1993). Yet, many questions about this species including its ecology, mating and extent, distribution and origin of its laterality remained uncertain or untested until now. How this asymmetrical head morphology evolved (Koblmüller et al., 2007), what bones are actually involved in bringing about the head asymmetry, and what the underlying genetic and/or environmental contributions are all questions that are still either completely unanswered or debated (Palmer, 2010; Stewart & Albertson, 2010; Van Dooren et al., 2010). Also how this mouth asymmetry manifests itself throughout the ontogeny of the fish remained unknown, although some progress on this issue has been made (Stewart & Albertson, 2010; Van Dooren et al., 2010). From an ecological standpoint it is still not fully understood how it is maintained in natural populations. Our study investigated the degree and distribution of mouth asymmetry in juveniles as well as adults, collected information on the relative abundance of morphs in wild caught individuals from several populations and tested previous hypotheses on mating preferences. Combined with our behavioral data on the interaction of foraging behavior and mouth asymmetry (Lee et al., 2012), we advance the understanding of the evolution and ecological maintenance of the interesting laterality in this species.

\textit{(a) Equal dimorphism and random mating between mouth morphs}

In five populations of \textit{P. microlepis} we found both R- and L-morphs to be equally abundant, confirming the results of previous investigations (Hori, 1993), in spite of their sampling sites being separated from ours by hundreds of kilometres of coastline.

Based on the largest field sample of mated pairs collected for \textit{P. microlepis} to date, we detected random mating with regard to laterality (figure 2). This contradicts previous reports based on smaller sample sizes, that claimed to have found disassortative mating (Takahashi & Hori, 2008). These new field data are consistent with our previous work that revealed no genetic differentiation between morphs based on both, mitochondrial and nuclear DNA markers, but that provided genetic support for
the random mating hypothesis (Lee et al., 2010). Hypothetical selective advantages for breeding assortatively or disassortatively were thus not reflected or detected by genetic analyses. The disagreement between our results and those of previous investigations (Takahashi & Hori, 2008) may be due to their use of an insufficient sample size. Alternatively, there might be heretofore unreported intraspecific variation in mating preferences among populations – instances of this are known from other vertebrates (Lott, 1991). Geographic aspects might play a role in mating variation, especially when considering that genetic isolation by (small) distances between populations of *P. microlepis* has been reported along rocky shorelines of southern Lake Tanganyika (Koblmüller et al., 2009; Lee et al., 2010). Although we observed a slightly higher number of disassortative pairs (*n* = 29) than expected by chance (*n* = 25.34), this difference was not statistically significant. A larger sample size might again reveal evidence for disassortative mating (Takahashi & Hori, 2008). However, even if such a slight deviation from random mating existed, it would not be likely to stabilize morph frequencies (Takahashi & Hori, 2008). The suggestion that phenotypic plasticity plays a large role in shaping mouth asymmetry in this species (Van Dooren et al., 2010), would indirectly support our findings of random mating with regard to laterality.

(b) *Trait variation in mouth asymmetry is not a discrete dimorphism*

We show that mouth asymmetry is continuously and unimodally (generally non-platykurtic) distributed in both adult and juvenile *P. microlepis*, which contrasts rather strongly with the previous reports about the discreteness and bimodality of this trait (Hori, 1993; Hori et al., 2007).

By quantifying mouth opening direction in a large number of individuals, we show that many juveniles and adults have virtually symmetrical mouths (Figure 21, Figure 22), which argues against the previous hypothesis that mouth laterality of *P. microlepis* is determined exclusively by two alleles at a single genetic locus (Hori, 1993; Hori et al., 2007; Stewart & Albertson, 2010). If that hypothesis were correct then symmetrical or near-symmetrical fish would not be expected to be present unless environmental variation also plays a role in the determination of mouth directionality (Lynch & Walsh, 1998).
Trait means, regardless of the measurement technique, were not different from zero in either adult or juvenile fish. Similarly, the ANOVA and MANOVA analyses of mouth asymmetry (Table 5, Table S6) generally favour non-directional asymmetry. Each specific data set (e.g. mouth bending angle, PC-1 score) most likely consisted of a single component (Table 4) and not two components as would be expected if the trait were strongly antisymmetric (Figure S8). However, in the pooled adult sample the test for platykurtosis in the distribution of mouth bending angle was nearly significant ($p = 0.06$) and in the laboratory-reared three month old fish platykurtosis in the distribution of PC-1 score was significant. Thus the possibility of weak AS cannot be excluded. On the whole, in the context of left-right asymmetry our data suggest that mouth asymmetry variation in this scale-eating cichlid fish is much more continuously distributed than previously believed and is not a discrete dimorphism at all. Consequently, if mouth laterality in *P. microlepis* has any genetic basis at all, it is weak and coupled with significant environmental input (Lynch & Walsh, 1998).

In spite of the general non-significant relationship, mouth asymmetry of adult *P. microlepis* is inclined to increase with body size in two sampling locations as well as in the pooled field sample (Table 4). This (weak) positive association would be expected if mouth asymmetry were to amplify over individual’s lifetime as a phenotypically plastic response to repeated attacks at a particular prey-side (Palmer, 2010; Van Dooren et al., 2010), assuming that behavioral handedness is not particularly plastic (Lee et al., 2012-Chapter V of the present thesis; Van Dooren et al., 2010). Platykurtosis was also marginally non-significant in the pooled sample. It is therefore conceivable that if our sampling included a greater number of larger fish, this positive trend and platykurtosis would become significant, which in turn leaves room for the possibility of weak dimorphic asymmetry.

Several further lines of evidence support the hypothesis that mouth asymmetry is not a discrete dimorphism. In a previous study, we (Lee et al., 2010) also found that 11 out of 128 (9 %) individuals of adult *P. microlepis* from two populations from southern Lake Tanganyika could not, based on visual inspection, be unambiguously assigned to a particular laterality morph. Similarly, more than 10 % (5 out of 47) adults from a northern population from Lake Tanganyika were also almost perfectly symmetrical (mouth bending angle less than 1°) (Van Dooren et al., 2010). The results
of (Van Dooren et al., 2010) further suggest that mouth asymmetry is difficult to assess without a quantitative measure of mouth asymmetry. Nevertheless many wild-caught adult individuals still have a mouth that bends strongly to the left or the right (Figure 21). However, our assessments of both pooled and population samples showed that variation in mouth asymmetry is unimodally distributed.

Further, even with the aid of a dissecting microscope, it was difficult to judge mouth laterality in the three month (~ 3-4 cm total length (TL)) and seven month (~ 8.0 cm in TL) old fish. This does not agree with previous reports that laterality is readily discernible in juvenile fish whose TL is greater than 1.2 cm (Hori et al., 2007). When mouth bending angle was estimated for juveniles of a single *P. microlepis* brood, mouth laterality could be confidently assigned for only 93 of 141 (66 %), while the mouths of the remaining 48 individuals were nearly symmetric (Stewart & Albertson, 2010). Thus, the frequency of mouth asymmetry in that study (Stewart & Albertson, 2010) of juveniles showed a unimodal trait distribution, which would not be the case if it were a discrete dimorphism.

In comparison to wild-caught fish, the rather symmetrical mouth morphology in our laboratory-reared seven month old juveniles lends indirect support to the hypothesis that phenotypic plasticity considerably contributes to mouth laterality (Van Dooren et al., 2010). This is because the fish were fed almost exclusively by *Artemia* nauplii and flake food, but they were not provided with prey fish that would allow them to feed on scales. One might speculate that they might have developed apparent asymmetry if they had the regular opportunity to prey on scales (Lee et al., 2012 - Chapter V of the present thesis).

We here document a generally unimodal and non-platykurtic distribution (with the mean not being different from zero) of mouth asymmetry. The measurements from photographs taken from a dorsal view suggest that the trait variation in mouth laterality of *P. microlepis* does not reflect a discrete dimorphism. We recognize that this type of measurements (e.g. mouth bending angle) may not fully capture the existing mouth laterality of this species. In the assessments of laterality in live *P. microlepis* one can open the mouths by pulling down the upper jaw. In most specimens the laterality of the mouth opening direction becomes much more apparent and can thus be less ambiguously scored, based on the relative orientation of the ascending process of the
premaxillary bone and the laterality of mouth opening direction. Our scoring of mouth asymmetry of live fish overwhelmingly fit their later foraging decisions (Lee et al., 2012 - Chapter V of the present thesis). We are currently investigating what role environmental (and behavioral) variation rather than genetic influences play in bringing about this pronounced foraging laterality and in mouth-opening morphology of this species.

Acknowledgements

Field research was conducted with study permit (G.R. No: 2077761) issued from the Department of Immigration of the Republic of Zambia to HK. Support was provided through the International Max Planck Research School (IMPRS) for Organismal Biology to HK, through the Zukunftskolleg postdoctoral fellowship, the Deutsche Forschungsgemeinschaft (DFG) (LE2848/1-1) and the Young Scholar Fund (FP 411/12) of the University of Konstanz to HJL and through grants of the Deutsche Forschungsgemeinschaft and the University of Konstanz to AM. We thank Christian Sturmbauer for support in the field. We thank Mr. Zymbo, Mr. Chiti, Mr. Chansa, Mr. Musosa and particularly Gabriele Legant for their superb technical assistance in the field. Thanks to Frederico Henning for statistical advice and Helen Gunter for her suggestions for improving the manuscript. We especially thank Tom Van Dooren and A. Richard Palmer for valuable comments on this manuscript.
Chapter V

Handed foraging behavior in scale-eating cichlid fish: its potential role in shaping morphological asymmetry

Hyuk Je Lee*, Henrik Kusche* & Axel Meyer

Published in *PLoS One* : 7 (9):e44670 (2012)

*equal contribution
Abstract

Scale-eating cichlid fish, *Perissodus microlepis*, from Lake Tanganyika display handed (lateralized) foraging behavior, where an asymmetric “left” mouth morph preferentially feeds on the scales of the right side of its victim fish and a “right” morph bites the scales of the left side. This species has therefore become a textbook example of the astonishing degree of ecological specialization and negative frequency-dependent selection. We investigated the strength of handedness of foraging behavior as well as its interaction with morphological mouth laterality in *P. microlepis*. In wild-caught adult fish we found that mouth laterality is, as expected, a strong predictor of their preferred attack orientation. Also laboratory-reared juvenile fish exhibited a strong laterality in behavioral preference to feed on scales, even at an early age, although the initial level of mouth asymmetry appeared to be small. This suggests that pronounced mouth asymmetry is not a prerequisite for handed foraging behavior in juvenile scale-eating cichlid fish and might suggest that behavioral preference to attack a particular side of the prey plays a role in facilitating morphological asymmetry of this species.
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Introduction

Within-species behavioral polymorphisms are ubiquitous. One particularly interesting case is handed (lateralized) behavior, where individuals exhibit a behavioral bias towards either one or the other side. The most familiar example of lateralized behavior comes from humans, where most individuals (89%) are right-handed and a minority is left-handed (8%) or ambidextrous (3%) (Vuoksimaa et al., 2009). Behavioral lateralization can also frequently be observed in other species; for example, hand-use preference in Chimpanzees (Hopkins & Leavens, 1998) and in some birds like Australian parrots (Brown & Magat, 2011), and foraging preference in the Japanese snail-eating snakes (Hoso et al., 2007; Hoso et al., 2010). This lateralized behavior, particularly in mammals and birds, is thought to be linked to lateralized brain functions and neuro-anatomical asymmetries (reviewed in Rogers & Andrew, 2002)). From an ecological perspective, handed behavior is suggested to have evolved because it might provide organisms with a selective advantage (e.g. in terms of foraging efficiency in the snail-eating snake, *Pareas iwasakii* (Hoso et al., 2007); escape performance from predator attacks in the shiner perch, *Cymatogaster aggregata* (Dadda et al., 2010); predation success in the scale-eating cichlid, *Perissodus microlepis* (Hori, 1993)).

Handed behavior has also been frequently reported in fish, e.g., with respect to eye usage preference (i.e. visual lateralization) in a poeciliid fish (Bisazza et al., 2007; Sovrano et al., 2005), swimming-turns in zebra-and goldfish (Heuts, 1999) and foraging in a freshwater goby (Seki et al., 2000) and in some African cichlid fishes (Hori et al., 2007; Mboko et al., 1998). Lateralized behavior in fish is often correlated with morphological asymmetries. In the herbivorous cichlid *Telmatochromis temporalis*, for example, the right mouth morph uses the right side of the jaw more frequently and the left morph the left side (Mboko et al., 1998). A significant correlation between lateralization in swimming and the anatomical bias of the prevalence of different muscle types was found in zebrafish (Heuts, 1999). However, relatively little effort has been directed towards the exploration of the potential role of handed behavior in facilitating morphological laterality (Palmer, 2012).

A well-known textbook example of a significant interaction between handed behavior and morphological laterality in fish is *Perissodus microlepis*, a scale-eating
(lepidophagous) cichlid fish species from Lake Tanganyika (Futuyma, 2009). This species is extremely ecologically specialized since most individuals either have a mouth that is bent to the left (‘L-morph’) or to the right (‘R-morph’) (see Figure 23), although a recent study suggests frequent occurrences of fish with a rather symmetrical mouth (Kusche et al., 2012 - Chapter IV of the present thesis).

![Figure 23. Dorsal view of left-bending right-bending mouth morphs of the Lake Tanganyikan scale-eating cichlid fish, *Perissodus microlepis*.](image)

L-morphs preferentially attack the right flanks of their prey fish while R-morphs attack the left flanks (Hori, 1993; Lee et al., 2010; Takeuchi et al., 2012; Van Dooren et al., 2010). This “lateralized (handed) foraging behavior” therefore represents an extreme form of specialization on a predominantly scale diet that is even restricted to scales from the left or the right side of prey fish. Yet, previous studies on lateralized scale-eating behavior of *P. microlepis* focused on adult fish only and the presence and strength of this lateralized behavior in juvenile fish have never been tested before. Therefore, how and when these behavioral preferences arise ontogenetically is unknown.

The frequency of L and R morphs in natural populations of *P. microlepis* is suggested to be maintained by negative frequency-dependent selection (Hori, 1993). Over time, the proportion of L and R morphs within populations oscillates around a 50:50 ratio (Hori, 1993; Lee et al., 2010). The resulting lateralized foraging behavior of
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*P. microlepis* is expected to make prey fish more alert to being attacked from the preferred side of the more abundant morph. Thus, increased prey vigilance would reduce the predation success of the more abundant morph, and negative frequency-dependent selection would thereby favor the rarer morph in each generation. Consequently, the frequency of both morphs is maintained in approximately equal abundances (Hori, 1993).

Several important questions about this fish, including the bases of its behavioral and morphological laterality remain unanswered (Palmer, 2010). Mouth laterality of *P. microlepis* has been suggested to be genetically determined by a single Mendelian locus with two alleles: the “R”-allele was suggested to be dominant over the “L”-allele and “R” was suggested to be homozygous lethal (Hori, 1993; Hori et al., 2007; Stewart & Albertson, 2010). But a recent review (Palmer, 2010) noted that the data reported so far (of mouth-morph ratios in the offspring of parents of known laterality) actually are inconsistent with a single locus Mendelian model. This model was further questioned because the distribution of mouth asymmetry was found to be unimodal rather than bimodal (Kusche et al., 2012 - Chapter IV of the present thesis; Stewart & Albertson, 2010; Van Dooren et al., 2010). Phenotypic plasticity may therefore play a role in shaping mouth asymmetry (Van Dooren et al., 2010) and hence, head asymmetry may be governed by both genetic and environmental factors (Van Dooren et al., 2010) (Lee *et al.*, unpublished data). The genetic and/or environmental basis of behavioral handedness in *P. microlepis*, however, remains largely unexplored.

Furthermore, whether foraging handedness is expressed earlier during development and induces and thereby facilitates mouth asymmetry via phenotypic plasticity (Palmer, 2012; Van Dooren et al., 2010) or the reverse – remains unclear. Hori (Hori, 1993) originally suggested that mouth laterality in *P. microlepis* is a functional “prerequisite” for efficient lepidophagy. He further proposed that mouth asymmetry (controlled by a single Mendelian locus) precedes and invokes and directs lateralized foraging behavior through natural selection. But, several lepidophagous cichlid species in Lake Tanganyika lack a pronounced laterality in their heads (Stewart & Albertson, 2010) and behavioral preferences have not been tested in these species.
Moreover, handed foraging behavior might actually precede, and even induce mouth asymmetry, given the purported role of phenotypic plasticity in mouth laterality (Van Dooren et al., 2010). In this study, we examine the strength and individual variation of lateralized behavior and its interaction with mouth laterality in *Perissodus microlepis*. In semi-natural conditions, we conducted feeding experiments on adult wild-caught scale-eaters with their natural prey to test whether pronounced morphological laterality predicts foraging preferences. We further tested whether laboratory-reared juvenile scale-eaters, which had never encountered prey fish before, displayed lateralized scale-feeding behavior in reference to mouth asymmetry. Here we demonstrate relatively strong handedness in foraging behavior in juvenile fish that showed much less mouth asymmetry (compared to wild-caught adult fish) and we then discuss the potential role of lateralized foraging behavior in shaping the head asymmetry of this species.

**Material and Methods**

**Sample collection**

Fifty-four breeding pairs of *Perissodus microlepis* were collected in April 2010 by diving with hand nets at Toby Veal’s Lodge (S08°37.4' E031°12') near Mpfungu (Zambia) on the southern tip of Lake Tanganyika to assess the mating pattern (Kusche et al., 2012 - Chapter IV of the present thesis). Twenty-one out of these 54 pairs were used for foraging experiments on adult *P. microlepis* under semi-natural conditions. Mouth laterality of each of the pairs was judged by eye in the field by two independent researchers (HK and AM) (see Figure 23).

Five broods from different parents of determined mouth-laterality (3 RL and 2 RR pairs) were transported to the animal care facility at the University of Konstanz. In total, 65 young were raised brood-wise in separate 40 l and later 200 l aquaria with *Artemia* nauplii and flake food. These fish were used for laboratory feeding experiments on juveniles as well as for quantitative measures of mouth asymmetry (Kusche et al., 2012- Chapter IV of the present thesis).

Sixty-one more juveniles from three different broods (3 young of RL, 6 of LL and 52 of RR parental-pairs) were obtained by breeding wild-caught fish in the laboratory. Lab-reared *P. microlepis* fish reached sexual maturity at about six to nine
months of age. These 61 juveniles, the F$_1$ fish of wild-caught stock, were used for foraging experiments that examined behavioral handedness and its interaction with mouth asymmetry.

Field research was conducted under the study permit (G.R. No: 2077761) granted by the government of the Republic of Zambia (Immigration Department, Ministry of Home Affairs, Republic of Zambia) according to their Immigration and Deportation Act. CAP123, Section 16. Animal care of the fish and all foraging experiments in the laboratory were approved by the regional board of animal welfare in Germany (Regierungspräsidium Freiburg, Abteilung Landwirtschaft, Ländlicher Raum, Veterinär- und Lebensmittelwesen) (permit number: 35/9185.81/G-10/96).

Field foraging experiments of adult fish
Thirteen outdoor pools (1000 l) at the shore of Lake Tanganyika were stocked with one breeding pair of *P. microlepis* each (7 RL, 5 RR and 1 LL pairs). Also two large community tanks of 4000 l volume (with 6 L-morphs and 10 R-morphs of *P. microlepis*, respectively) were used in these foraging experiments. The cichlid species *Tropheus moorii* (pair tanks: $n = 3 – 6$; community tanks: $n = 18$ and 25) was used as prey since it is a preferred natural prey species of *P. microlepis* (Nshombo et al., 1985). After 72 hours, all *T. moorii* were removed from the pools and presence/absence of scars and missing scales and the numbers of bites on both flanks of the prey fish were recorded.

Percentages of pooled attacked left (and right) flanks of prey fish were calculated for each tank. We considered these estimates as foraging preference for a particular side, given that the scale-eaters had in principle an equal opportunity to attack both flanks. Foraging preference was further assessed by taking into account foraging scores reflecting different levels of injury (i.e. the amount of damage done by the scale-eaters; 0 bites = 0 scores; 1 – 3 bites = 1 score; 4 – 6 bites = 2 scores; > 6 bites = 3 scores). The proportion of these scores for the prey fishes’ pooled left (and right) flanks was calculated for each tank.

Two-tailed Fisher’s exact probability tests were performed to examine whether in both community tanks the left or right side of the prey fish were preferentially attacked and whether the morphs differed in their foraging scores. A Mann-Whitney-
test was performed to test for differences in ratios of affected left flanks of the prey as well as in the amount of foraging scores between RL and RR pairs.

**Laboratory foraging experiments of juvenile fish**

Juvenile *P. microlepis* of about two \([n = 61; \text{mean standard length (SL)} = 3.2 \text{ cm}; \text{SD} = 0.28 \text{ cm}]\), three \([n = 47; \text{total length (TL)} = 3 – 4 \text{ cm}]\) and seven \([n = 24; \text{mean total length (TL)} = 7.7 \text{ cm}; \text{SD} = 0.58 \text{ cm}]\) months of age, that had not had an opportunity to eat scales from prey fish before, were tested for lateraled foraging behavior. The older test cohorts (that were tested at three and seven months) were caught as 1-2 week old fry in the field, whereas the fish tested at two months were bred in the laboratory (the F\(_1\) fish of the older cohorts; see above). Eleven of the three-month old fish were re-tested at seven months, but those individuals could not be traced due to logistical reasons. The scale-eaters were placed individually with a single prey fish (platy fish, *Xiphophorus maculatus*, for three-month old fish and goldfish, *Carassius auratus auratus*, for two- and seven-month old fish) in the trial tanks.

Two different methods were used to analyze foraging behavior. For the three-month old fish, one prey fish was added to a 40 \(l\) aquarium and after 12 hours, the prey fish was examined for scars and missing scales by two different researchers (HL and HK). This procedure was replicated (2-5 times) for each individual scale-eater to investigate whether its foraging behavior was consistent across a series of 2-5 experimental trials during a period of 1-2 weeks. Because it was impossible to enumerate number of scars and missing scales on the prey fish (*X. maculatus*), foraging preference was assessed for each scale-eater based on observed presence/absence of scars and missing scales. A foraging score of + 1 was given for fish that attacked only the right side of prey fish in a particular trial, a score of 0 meant that both sides were attacked, and a score of − 1 was given for fish that attacked only the left side. The trials where no scars and no missing scales were observed on the prey fish, or where the prey fish died during the experiments were excluded from the analysis. Since the estimated foraging score of each individual was found to be constant over the trials (e.g. 2 trials: Wilcoxon-signed-ranks-test, \(n = 17, z = − 0.333, p = 1.0\); 3 trials: Friedman-test, \(n = 12, \chi^2 \text{ statistic} = 4.333, p = 0.189\)), the mean foraging score was calculated and used in further analyses. Note that the foraging score of 34 scale-eaters was calculated from the 2-5 trials, while that of 10 individuals was obtained from a single trial only.
Only three of the 47 scale-eaters (6 %) tested at three months never fed on scales.

To more precisely quantify “behavioral” foraging preference in juvenile *P. microlepis*, a second series of experiments for the seven- and later two-month old scale-eaters was carried out. For each seven-month old individual, its foraging behavior was monitored (in 3-4 replicates during 1-2 weeks) by counting the number of attacks to the left and/or right flanks on a single goldfish, until a total of maximally 20 attacks per individual within up to 30 minutes were reached. The scale-eaters showed reported natural foraging behavior, i.e., they attacked prey from behind (Hori, 1993). In only a few cases they attacked from the front, but those attacks were not counted. Behavioral foraging preference (i.e. probability of left attack) was again found to be consistent among the 3-4 trials (repeated-measure ANOVA; $F_{3, 45} = 0.363, p = 0.78$) as observed in the three-month old fish. Therefore, the handedness scores (e.g. number of left and right attacks) were pooled over the trials to calculate behavioral foraging preference for each scale-eater. The total number of attacks observed per fish ranged from 39 to 80 (mean = 64). For the two-month old scale-eaters, we employed the same procedure as for the seven-month old fish, except that we conducted only one experimental trial per individual. The average number of attacks observed per fish in this test cohort was 19.

To statistically analyze if juvenile *P. microlepis* fish show a bimodal or unimodal distribution in their foraging behavior (e.g. foraging score, behavioral foraging preference), the dip statistic (Hartigan & Hartigan, 1985) and a mixture analysis with a parametric bootstrap test (1000 iterations) using the mixtools package (Benaglia et al., 2009) were performed in R (R Core Team, 2012). An Anscombe-Glynn test (Anscombe & Glynn, 1983) for platykurtosis was further performed for the seven-month old fish only (see below).

To investigate whether behavioral foraging preference is translated into foraging score (e.g. number of scales bitten by the scale-eaters), surface areas of attacked left and right flanks of prey (i.e. surface areas of scars and missing scales) were calculated for a sub-sample ($n = 15$) of the two-month old fish. Because individual fish that exclusively attacked one side of the prey only left scars/missing scales at that flank (100 %), the 15 fish were selected from individuals that did not forage exclusively from one side (e.g. $0.1 < \text{probability of left attack} < 0.9$). The attacked areas of the prey fish were estimated in ImageJ 1.45r (http://imagej.nih.gov/ij) from standardized photographs in a lateral
view with an implemented scale. A ratio of the attacked areas (left to right flanks) on
the prey fish was calculated for each scale-eater and linear regression analysis was then
conducted using probability of left attack as an independent variable (predictor) and the
estimated ratio as a dependent (response) variable.

**Relationship between mouth laterality and behavioral handedness in juveniles**

The mouth bending angle, “α L − β R” in ° following (Hori et al., 2007) was measured
to test for a relationship between mouth/head asymmetry and handed foraging behavior
in juvenile fish. For this test, each live test fish was photographed from a dorsal view in
a standardized upright position using a Zeiss Axiophot digital microscope (Zeiss,
Germany). The mouth bending angles were then measured in ImageJ 1.45r: on each
image, a triangle connecting the most anterior points of the eye sockets and the tip of
the snout was drawn to estimate angles (°), α L (angle of the vertex by the left eye) and
β R (angle of the vertex by the right eye) (Kusche et al., 2012- Chapter IV of the present
thesis).

To evaluate the accuracy of the measurements, repeatability of α L − β R was
estimated from repeated and blind measurements that were done from two replicate
photographs of the same individuals from sub-samples (n = 20, 15 and 15 for the two-,
three- and seven-month old fish, respectively). Repeatability, referred to as the
proportion of the total variation that is due to variation among individuals, was
calculated from one-way ANOVA (individual = factor) following (Sokal & Rohlf,
1995).

Correlation analyses were performed between mouth bending angles and
foraging score (for our test cohort of the three-month old fish) and behavioral foraging
preference (probability of left attack for the two- and seven-month old fish) to test for
the significant relationship between mouth asymmetry and behavioral handedness.
Linear regression analyses were also carried out to test whether mouth asymmetry
amplifies as body size increases in the two- and seven-month old fish. For those
analyses, SL (standard length) and TL (total length) were used as size measures for the
two- and seven-month old fish, respectively.
Results

Mouth laterality predicts preferred attack side in adult fish

Mouth laterality strongly predicted the preferred attack side as well as foraging scores on either side of the prey fish (Figure 24, Table S7). Both L- and R-mouth morphs from the community tanks clearly exhibited opposed foraging preferences (Fisher’s exact probability test: $n = 41; p < 0.001$) and yielded more scars/missing scales in foraging from their preferred flanks (Fisher’s exact probability test: $n = 63; p < 0.0001$) (Figure 24A-B). R-morphs preferentially attacked left flanks of the prey fish (80% of affected flanks; 82% of foraging scores). L-morphs preferred to feed from right flanks (75% of affected flanks; 80% of foraging scores).

Figure 24. Lateralized foraging behavior in adult *Perissodus microlepis.*

Mouth asymmetry strongly predicts foraging preferences and foraging scores in community tanks (A and B) and pair tanks (C and D) of different laterality combinations.
The same clear pattern was found in the 13 pools with one pair of *P. microlepis* each as predators (Figure 24 C-D): seven RL pairs fed from both flanks with similar frequencies (ratio of attacked left flanks: 40 – 60 %; average: 52 %; median: 50 %) and produced similar amount of damage onto both flanks (range of foraging scores on left flanks: 25 – 67 %; average: 48 %; median: 50 %). Five RR pairs strongly preferred to feed from the left flank (range: 67 – 100 %; average: 88 %; median: 100 %), which caused more bites on that flank (range: 83 – 100 %; average: 94 %; median: 100 %). A single LL pair exclusively fed from the right flanks of their prey fish. Differences in foraging patterns such as foraging preference and foraging score among morph pair combinations were both highly statistically significant (Wilcoxon-rank-sum-test with continuity correction: proportion of left flanks affected: \( w = 35, p < 0.01 \); proportion of foraging scores at the left flank: \( w = 35, p < 0.01 \)).

**Strong handed foraging behavior in juvenile fish**

![Graphs showing foraging behavior](image)

*Figure 25. Lateralized foraging behavior in juvenile *Perissodus microlepis*.*

Frequency distribution of behavioral foraging preference (for two- and seven-month old fish) and foraging score (for three-month old fish) shows a bimodal distribution. (A) two month; (B) three month; (C) seven month old fish. In (C), the graphical inspection of the mixture analysis (fitting two single-component normal distributions to the data) is shown, indicating that the distribution better fits to bimodality than to unimodality, despite a marginal statistical significance of one single-component normal distribution \( p = 0.057 \).
Feeding experiments with laboratory-raised juveniles showed that nearly all test fish preyed immediately on scales. Scale-eating behavior is already expressed at an early ontogenetic stage (two-month old: 100%; three-month old: 94%; seven-month old: 100%). Most individuals showed a clear bias to attack only a particular side of their prey and the frequency distribution of the foraging score and behavioral foraging preference clearly exhibited a bimodal distribution (except in the seven-month old fish) (Figure 25). Foraging behavior of the younger test cohorts (of two and three months of age) showed a significant departure from a unimodal distribution (two-month old: dip statistic = 0.114, \( p < 0.001 \); three-month old: dip statistic = 0.136, \( p < 0.001 \)), whereas the oldest cohort of seven months of age did not (dip statistic = 0.057, \( p > 0.5 \)). The mixture analyses with the parametric bootstrap tests further showed that two-component normal distributions best fitted foraging behavior data of the two- (\( p < 0.001 \)) and three-month old fish (\( p < 0.001 \)), while one-component normal distribution statistically best fitted the data of the seven-month old fish with marginal significance (\( p = 0.057 \)). However, the graphical inspection of the mixture analysis (Figure 25C) and a marginal significance of platykurtosis (\( p = 0.092 \)) rather support a weak bimodal distribution (Palmer & Strobeck, 1992).

There was considerable “inter-individual” variation in the intensity (strength) of lateralized foraging behavior (Figure 25). Many fish strongly preferred or even exclusively attacked the left or the right sides [e.g. 7 of 61 (12%) and 15 of 61 (25%) of the two-month old fish foraged exclusively from the left and the right sides, respectively], while other fish displayed a less pronounced bias in foraging behavior (Figure 25).

As predicted, a highly significant positive correlation was found between behavioral foraging preference and foraging score in the subset (\( n = 15 \)) of the two-month old juveniles (\( y = 1.787x + 0.176, R^2 = 0.759, p < 0.001 \); Figure 26), suggesting that foraging score is an outcome of behavioral attack preference. This result further indicates that our field data on foraging preference and foraging score of wild-caught adult fish could indeed reflect “behavioral” foraging preference.
Figure 26. Relationship between behavioral foraging preference and foraging score.

Lateralized foraging behavior and foraging score (e.g. number of scales eaten by the scale-eaters) are highly significantly correlated (in a sub-sample \( n = 15 \) of the two-month old fish \( y = 1.787x + 0.176, R^2 = 0.759, p < 0.001 \)).

Lack of correlation between handed behavior and mouth asymmetry in juveniles

Our measurements of mouth bending angles appeared to be fairly repeatable: estimated repeatability of the mouth bending angles was 0.80, 0.77 and 0.87 for the two-, three- and seven-month old fish, respectively. Those estimates of the repeatability imply that 77 to 87 % of the total observed variation is attributed to underlying “true” variation in the mouth bending angles among individuals and the remaining 13 to 23 % variation is due to measurement error.
Figure 27. Relationship between mouth asymmetry and lateralized foraging behavior.

Mouth asymmetry (mouth bending angle) is not significantly correlated with foraging handedness in juvenile *P. microlepis*. (A) two month (*r* = 0.148; *p* = 0.255); (B) three month (*r* = −0.229; *p* = 0.154); (C) seven month old fish (*r* = 0.069; *p* = 0.749). Note that 11 fish were tested at two different ontogenetic stages (i.e. at three and seven months of age).

Neither of the juvenile cohorts showed a significant correlation between mouth asymmetry and lateralized foraging behavior (two-month old fish: *r* = 0.148; *p* = 0.255; three-month: *r* = −0.229; *p* = 0.154; seven-month: *r* = 0.069; *p* = 0.749; Figure 27). Unexpectedly, some fish that were morphologically scored as (slightly) R-morphs (with negative values of mouth bending angle of αL − βR) occasionally even attacked the right side more frequently than the left side, and vice versa (Figure 27). This lack of correspondence suggests that mouth laterality is not a prerequisite for handed foraging behavior for juvenile *P. microlepis*. Also, the level of mouth asymmetry (i.e. absolute values of mouth bending angles) of the laboratory-reared juvenile fish did not significantly increase with body size in either two-month (*n* = 61, *y* = 0.831x + 0.04, *R*² = 0.015, *p* = 0.35) or seven-month (*n* = 24, *y* = −0.496x + 5.394, *R*² = 0.032, *p* = 0.4) old fish.

**Discussion**

The handedness of the foraging behavior and the associated asymmetry in mouth/head morphology have made the scale-eating cichlid fish, *Perissodus microlepis*, a textbook example (Futuyma, 2009) of both, the astonishing degree of ecological specialization and negative frequency-dependent selection (Hori, 1993). However, how and when
lateralized foraging behavior manifests itself during ontogeny, and whether its association with mouth asymmetry is already apparent in juvenile individual fish had remained untested. Here, we report on the strength and individual variation of lateralized foraging behavior as well as its relationship with mouth asymmetry in *P. microlepis* during its juvenile as well as adult life stages. We find that handed foraging behavior is already prominent at an early age (e.g. at two-months), although the initial morphological asymmetry is less evident, which hints that handed behavior might play a role in bringing about pronounced morphological laterality, considering the potential influences of phenotypic plasticity on mouth asymmetry (Van Dooren et al., 2010).

The observed strong lateralization in foraging behavior in young scale-eaters (e.g. bimodal distribution) that was not accompanied by notable morphological asymmetry (Kusche et al., 2012- Chapter IV of the present thesis) and the obvious correspondence between mouth orientation and foraging behavior in adult fish might suggest that handed behavior is probably expressed earlier during development. And, it may actually induce and facilitate morphological asymmetry (Van Dooren et al., 2010), if phenotypic plasticity plays a relatively larger role than the genetic determination of this trait (Lee *et al.*, unpublished data). An alternative hypothesis is that both handed behavior and morphological laterality are genetically governed, but expressed at different ontogenetic stages. However, this hypothesis would seem to be rather unlikely given our observation that laboratory-reared fish of now about two-year of age still have a relatively symmetrical mouth (HL, personal observation).

The field foraging experiments with adult fish clearly demonstrate that manifested mouth laterality corresponds to a pronounced bias in feeding laterality. This finding is consistent with previous studies that discovered strong correlations between mouth laterality and handed foraging behavior in adult fish (Hori, 1993; Lee *et al.*, 2010; Takeuchi *et al.*, 2012; Van Dooren *et al.*, 2010). However, not every adult individual exclusively attacked the prey flank according to its scored mouth laterality (see Figure 24, Table S7 in the current study and Lee *et al.*, 2010).
The observed level of individual variation in the degree of lateralized foraging behavior might translate into varying expression of mouth asymmetry (Kusche et al., 2012-Chapter IV of the present thesis), provided – and this may be a strong assumption – that mouth laterality, but not foraging preference are appreciably influenced by environmental factors (Van Dooren et al., 2010). The observed consistency of foraging behavior in the three- and seven-month old juveniles over the repeated experiments during 1-2 weeks supports the hypothesis that handed behavior is not particularly plastic (over that time-scale), which is consistent with the previous study (Van Dooren et al., 2010). How much of the observed morphological differences are initially brought about by a (heritable?) behavioral bias that, through phenotypic plasticity becomes also fixed on a morphological level remains to be tested.

However, we observed a rather more pronounced laterality in foraging behavior among the younger juvenile fish (e.g. at two and three months), compared to the fish at seven months. The observed dwindling laterality in foraging behavior in the older fish might imply that feeding preference is expressed at an early age (e.g. at two months), but the initial level of laterality would diminish over time (under laboratory conditions) unless the fish were constantly to feed on scales. Yet, whether this trend means the strength of handed behavior truly decreases with age awaits future experiments on “tracked” individuals over a series of ontogenetic stages during their life time.

The foraging experiments with juvenile fish in the laboratory show that mouth asymmetry does not predict handedness in foraging behavior, possibly due to the small degree of mouth asymmetry. Surprisingly, young and still quite small scale-eaters preyed on scales of prey fish and exhibited pronounced handed behavior (Figure 25). Even the two-month old fish readily fed on scales of similar- or even slightly larger-sized goldfish. However, the degree of mouth asymmetry in juvenile scale-eaters was rather small [on average only 2.67° (for two-month: SD = 1.92°), 2.01° (three-month: SD = 1.73°) and 2.12° (seven-month: SD = 1.61°)] and the relationship between behavioral bias and morphological asymmetry was always non-significant (Figure 27). Note that the degree of mouth asymmetry in those laboratory-reared scale-eaters is indeed substantially lower than in wild-caught adult *P. microlepis* (the average = 5.07°; n = 238; SD = 3.51°; (Kusche et al., 2012-Chapter IV of the present thesis).
This too supports the hypothesis that handed behavior might play a significant role in shaping the asymmetry of mouths in *P. microlepis*.

Nonetheless, the observed lack of correlation between mouth asymmetry and lateralized foraging behavior in juvenile *P. microlepis* might also result from the measurement technique used for the quantification of mouth asymmetry (i.e. mouth bending angle) not fully capturing the existing true laterality (i.e. asymmetric skeletal features) in the mouth/head apparatus of this fish. Further tests with cleared and double-stained juvenile fish samples of known behavioral laterality are required to check this possibility.

We here argue that the hypothesis – “handed behavior preceding and driving mouth asymmetry” (Palmer, 2012; Van Dooren et al., 2010) – seems more strongly supported by evidence than the original hypothesis (Hori, 1993) that mouth asymmetry precedes and directs lateralized foraging behavior through natural selection acting on a single gene. Different lines of evidence support this hypothesis. In a parallel study, we observed a large amount of variation in mouth asymmetry in 238 wild-caught adult specimens and found a continuous and unimodal (and not bimodal) trait distribution (Kusche et al., 2012- Chapter IV of the present thesis). If plasticity rather than genetics plays a comparatively larger role (Lee *et al.*, unpublished data), then this unimodal distribution of laterality might simply be the outcome of different levels of lateralization in foraging behavior. This hypothesis is supported by the findings from the foraging experiments of juvenile fish: juvenile fish did not show complete lateralization of foraging preference and even some individuals attacked equally often at both flanks. Whether symmetrically attacking fish might have a potential selective advantage over left or right preferentially attacking fish needs to be tested.

We have indirect evidence that phenotypic plasticity has an influence on mouth asymmetry. The observed relatively symmetrical mouth morphology of the laboratory-reared fish even at the age of seven months (the degree of mouth angles is similar to the two- and three-month old juveniles; see above), would lend support to the hypothesis that phenotypic plasticity considerably contributes to the mouth laterality (Van Dooren *et al.*, 2010), because the fish did not have prior opportunities to feed on scales, but were fed almost exclusively on regular flake food. It seems that mouth asymmetry could not manifest itself under the laboratory condition with regular food (HL, personal
observation). The lack of positive association between the level of mouth asymmetry and body size in the laboratory-reared juvenile fish further supports this hypothesis. By comparison, in wild-caught adult fish mouth asymmetry tends to increase with size (Kusche et al., 2012- Chapter IV of the present thesis), which would be expected if mouth asymmetry were to amplify over an individual’s lifetime as a phenotypically plastic response to repeated attacks from one particular side (Palmer, 2012; Van Dooren et al., 2010).

A significant role of phenotypic plasticity in the evolutionary origin of novel morphologies has been suggested repeatedly during the last several decades (Greenwood, 1965; Moczek et al., 2011; Palmer, 2012; Wcislo, 1989; Wimberger, 1994). Phenotypic plasticity clearly contributes to shaping the morphology of the jaw and the mouth apparatus in teleost fishes, particularly in cichlids (Meyer, 1987; Wimberger, 1991). Even different food types or diet hardness can induce changes in the external shape of the head during the ontogeny of some cichlids (Meyer, 1987; Muschick et al., 2011). The teleost skeleton can quickly adapt to changing external factors, so called “mechanical adaptation”, and skeletal phenotypic plasticity in teleosts seems to be rather pronounced and taxonomically widespread (Witten & Huyseune, 2009).

Through phenotypic effects of “use and disuse”, handed behavior has been shown to drive morphological laterality in different animal groups (e.g. lobsters (Govind & Pearce, 1986); snakes (Hoso et al., 2007; Hoso et al., 2010); humans (Pearson & Lieberman, 2004)) (reviewed in (Palmer, 2012)). Lobsters provide a clear example of how claw asymmetry is shaped during development as a function of handed behavior (Govind & Pearce, 1986). Laboratory experiments demonstrated that differential use of claws during early juvenile stage induces and facilitates development of a crusher claw (Govind & Pearce, 1986). As such, in P. microlepis lateralized behavior might conceivably lead to an asymmetric remodeling of the structural elements (e.g. bones) involved in defining mouth shape (Ruff et al., 2006), given that lateralized behavior in fish sometimes has a strong additive genetic component (Bisazza et al., 2000; Brown et al., 2007), e.g., the estimated heritability of laterality of eye preference in the poeciliid fish, Girardinus falcatus is 0.5 to 0.6) (Bisazza et al., 2000). Although we are uncertain whether handed scale-eating behavior is genetically programmed
(innate), rather than environmentally plastic (learning) or both (Tierney, 1986), the bimodal trait distribution in very young fish (Figure 25) speaks for a major genetic locus determining handedness in scale-eating behavior (Van Dooren et al., 2010).

In a broader context, our study provides information on a long-standing controversy over the role of behavior in facilitating developmental morphological changes in an adaptive direction (e.g. phenotypic accommodation (West-Eberhard, 2003; West-Eberhard, 2005)) and in subsequent evolution of novel forms (e.g. (Bertossa, 2011; Palmer, 2012)). If behavior-induced morphological innovations provide an organism with improved performance in a given environment, such a behavioral response may secure an individual’s survival and reproduction and thus direct available variation in the following generation if traits involved in this behavioral response or the responsiveness per se are heritable (Baldwin, 1896). Handed behavior-induced mouth laterality in *P. microlepis* is believed to be functionally significant and selectively advantageous (in regard to feeding success (Takeuchi et al., 2012)). This line of thinking is reminiscent of C.H. Waddington’s ideas about “genetic assimilation” (Waddington, 1953), an idea that is in line with recent findings in epigenetics and might merit renewed attention and research effort.

**Acknowledgements**

We thank Christian Sturmbauer for support in the field. Thanks to Frederico Henning for statistical advice and Julia C. Jones, Tom J.M. Van Dooren and Richard A. Palmer for helpful comments on an earlier version of the manuscript. We thank Mr. Zyambo, Mr. Chiti, Mr. Chansa, Mr. Musosa and particularly Gabriele Legant for their superb technical assistance in the field.
Record of Related Contributions

1. Local variation and parallel evolution: morphological and genetic diversity across a species complex of Neotropical crater lake cichlid fishes

This study by Kathryn R. Elmer, Henrik Kusche, Topi K. Lehtonen & Axel Meyer was published in the *Philosophical Transactions of the Royal Society, B* 365 (1547): 1763-1782 (2010) and is a review of the evolutionary history and relevance of the Midas cichlid species complex, along with presenting morphological diversity.
Abstract: The polychromatic and trophically polymorphic Midas cichlid fish species complex (*Amphilophus cf. citrinellus*) is an excellent model system for studying the mechanisms of speciation and patterns of phenotypic diversification in allopatry and in sympatry. Here, we first review research to date on the species complex and the geological history of its habitat. We analyze body shape variation from all currently described species in the complex, sampled from six crater lakes (maximally 1.2–23.9 kyr old) and both great lakes in Nicaragua. We find that Midas cichlid populations in each lake have their own characteristic body shape. In lakes with multiple sympatric species of Midas cichlid, each species has a distinct body shape. Across the species complex, most body shape change relates to body depth, head, snout and mouth shape and caudal peduncle length. There is independent parallel evolution of an elongate limnetic species in at least two crater lakes. Mitochondrial genetic diversity is higher in crater lakes with multiple species. Midas cichlid species richness increases with the size and age of the crater lakes, though no such relationship exists for the other syntopic fishes. We suggest that crater lake Midas cichlids follow the predicted pattern of an adaptive radiation, with early divergence of each crater lake colonization, followed by intralacustrine diversification and speciation by ecological adaptation and sexual selection.

2. Two new endemic species in the Midas cichlid species complex from Nicaraguan crater lakes: *Amphilophus tolteca* and *Amphilophus viridis* (Perciformes, Cichlidae)

This study by Hans Recknagel, Henrik Kusche, Kathryn R. Elmer & Axel Meyer is currently under review in *AQUA, International Journal of Ichthyology* and describes two new Midas cichlid species based on eco-morphological and population genetic analysis.

Abstract: The Neotropical Midas species complex (*Amphiophus citrinellus* Günther) of cichlid fishes has become a model system for investigating the mechanisms of speciation, and the formation of adaptive radiations. A number of ecologically,
morphological, and genetically differentiated Midas cichlid species have already been identified in allopatry and in sympatry in this geographic setting of the chain of Nicaraguan crater lakes that form a “natural experiment”. Here, two new endemic Nicaraguan crater lake cichlids species are described: *Amphilophus tolteca* sp. nov., a slender-bodied species which is endemic to the young lake Asososca Managua and *Amphilophus viridis* sp. nov., an endemic benthic species from Lake Xiloá. *A. tolteca* morphologically resembles previously described limnetic species from the crater lakes Apoyo and Xiloá with a depressed, elongated body. However, *A. tolteca* is geographically isolated and genetically distinct from those and from the putative generalist ancestral species. *A. viridis* resembles the Xiloá species *A. amarillo* in terms of body shape, but is distinct in coloration, ecology and genetically differentiated from all other syntopic species.

3. Parsing parallel evolution: ecological divergence and differential gene expression in the adaptive radiations of thick-lipped Midas cichlid fishes from Nicaragua

This study by Tereza Manousaki, Pincelli M. Hull, Henrik Kusche, Gonzalo Machado-Schiaffino, Paolo Franchini, Chris Harrod, Kathryn R. Elmer & Axel Meyer was published in *Molecular Ecology* 22 (3):650-69 (2013). The study investigates the putative parallel evolution of Midas cichlids with enlarged lips throughout the species complex and uses a combined approach of morphological, genetic and diet analysis.

**Abstract:** The study of parallel evolution facilitates the discovery of common rules of diversification. Here, we examine the repeated evolution of thick lips in Midas cichlid fishes (the *Amphilophus citrinellus* species complex) - from two Great Lakes and two crater lakes in Nicaragua- to assess whether similar changes in ecology, phenotypic trophic traits and gene expression accompany parallel trait evolution. Using next-generation sequencing technology, we characterize transcriptome-wide differential gene expression in the lips of wild-caught sympatric thick- and thin-lipped cichlids from all four instances of repeated thick-lip evolution. Six genes (apolipoprotein D, myelin-associated glycoprotein precursor, four-and-a-half LIM domain protein 2, calpain-9,
GTPase IMAP family member 8-like and one hypothetical protein) are significantly underexpressed in the thick-lipped morph across all four lakes. However, other aspects of lips’ gene expression in sympatric morphs differ in a lake-specific pattern, including the magnitude of differentially expressed genes (97-510). Generally, fewer genes are differentially expressed among morphs in the younger crater lakes than in those from the older Great Lakes. Body shape, lower pharyngeal jaw size and shape, and stable isotopes and δ¹³C and δ¹⁵N differ between all sympatric morphs, with the greatest differentiation in the Great Lake Nicaragua. Some ecological traits evolve in parallel (those related to foraging ecology; e.g. lip size, body and head shape) but others, somewhat surprisingly, do not (those related to diet and food processing; e.g. jaw size and shape stable isotopes). Taken together, this case of parallelism among thick- and thin-lipped cichlids shows a mosaic pattern of parallel and nonparallel evolution.
General Conclusions

It is their exuberant biological diversity in terms of eco-morphological specialization, coloration and behavior that makes cichlid fishes a paradigm of evolutionary success. More than 2,000 species have been described to date, making them the most species-rich family of vertebrates. Cichlid fishes represent convincing examples of adaptive radiation and parallel evolution. Consequently, when addressing the origin and maintenance of such stunning biodiversity, cichlids warrant consideration among other suitable study systems to investigate these central questions in biology. Evidently, such major questions can be tackled by various approaches and I am most interested in finding answers by investigating the phenotype and its interactions with the environment.

It is believed that partly due to their unparalleled polymorphic nature in phenotypic traits, cichlids have extensively diversified while other lineages of freshwater fishes did not to that extent. Plentiful work has been accumulated to validate the potential role of trophic polymorphisms in the maintenance and ongoing diversification in this group. One striking example is the adaptive diversification along the benthic-limnetic axis, which is also prevalent in many other taxa of lacustrine freshwater fishes, where high-bodied morphs (benthic) are usually associated with the shore habitat, whereas the slender and fusiform morphs (limnetic) occur in the open water column. Although, speciation events along this axis have evidently occurred in cichlids (e.g. from the East African Great Lakes), putative early stages of (polymorphic) divergence, have been barely investigated so far. Such early stages of divergence probably constitute the most appropriate context to understand the diversification process per se, i.e. the mechanisms and favorable environmental conditions leading to divergence. Chapter III deals with one such putative case of early divergence in the Midas cichlid system and it is concluded that character release, likely triggered from strong intraspecific competition for limited resources might be a first stage in the evolution of benthic-limnetic resource polymorphisms in the depauperate crater lake environment.
General Conclusions

Such an increased level of phenotypic variation and individual-based differential resource that is observed within this young crater lake population might in older crater lake replicates have led to the evolution of discrete resource polymorphisms along the benthic-limnetic axis, and possibly to speciation.

Although sex-linked color polymorphisms have been extensively studied in cichlids, mostly in the framework of sexual selection, the putative role of sex-independent color polymorphisms on parallel lineage diversification has only been vaguely addressed. This is probably due to several reasons including the rarity of sex-independent color polymorphisms in cichlids, the often complex genetic architecture of many color polymorphic traits, and the lack of evolutionary replicates. In the Midas cichlid system, a color polymorphism, which is not limited by these criteria, occurs. Some related ecological and evolutionary consequences are addressed in Chapters I & II. It is shown that color morphs throughout the Midas cichlid species complex are consistently ecologically differentiated. Importantly, the revealed genetic correlation of eco-morphology and color has an innate potential to facilitate sympatric diversification processes, given the link between the traits that are likely subject to divergent selection (eco-morphology) and those related to mate choice (color), and therefore to reproductive isolation. In a laboratory experiment, color-differential predation is demonstrated for a major predator of Midas cichlids (Chapter II). Altogether, it is suggested that the maintenance of the color polymorphism in general, and the relative low abundance of gold morphs across populations in particular, might be partly attributed to color-associated differential ecological niche use and to color-differential predation.

Chapters IV & V relate to the scale eating cichlid fish, Perissodus microlepis from Lake Tanganyika. A comprehensive quantitative investigation of this polymorphism and its elements was due, given recent debates surrounding this textbook example of a trophic polymorphism. In summary, a model, more complex than previously believed, is suggested. However, some challenges still remain that relate to the relative contributions of genetic and environmental influences in shaping morphological and behavioral lateralization in this system.
As a whole, ecological aspects of diversification based on trophic- and color polymorphisms in cichlid fishes are addressed throughout this thesis. Several aspects regarding the maintenance of such discontinuous phenotypic variation are addressed and the question is tackled how this might eventually contribute to ongoing diversification. Overall, most results obtained match with established theory. Albeit, so far undiscovered patterns of variation and differentiation are also detected, some of which might be considered as innovative findings. Hence, in the awareness of its limitations, this thesis shall add to the understanding of biodiversity and its origins, in particular in the framework of adaptive radiation.
Record of Achievements

Chapter I - A conspicuous color polymorphism facilitates diversification in a young adaptive radiation
AM & KRE proposed the study. All authors participated in the design of the study and in the collection of specimens. HK conducted the experiments, analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

Chapter II - Color-differential predation by a visually hunting predator and its potential implications for a young adaptive radiation of crater lake cichlids
AM and HK conceived and designed the study. HK conducted the experiment, analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

Chapter III - Individual specialization in an extremely young adaptive radiation of Nicaraguan crater lake cichlid fishes reflects ecological release
AM, KRE & HK conceived the study. All authors participated in the design of the study and in the collection of specimens. HK collected most of the data, performed the statistical analysis and drafted the manuscript. All authors read and approved the final manuscript.

Chapter IV - Mouth asymmetry in the textbook example of scale-eating cichlid fish is not a discrete dimorphism after all
All authors conceived and designed the study. HK and AM collected the field data. HK and HL performed the experiments in the laboratory. HK and HL analyzed the data. HK and HL drafted the paper. All authors revised and contributed to the final version of the manuscript.

Chapter V - Handed foraging behavior in scale-eating cichlid fish: its potential role in shaping morphological asymmetry
All authors conceived and designed the study. HK and AM performed the experiments in the wild. HL performed the experiments in the laboratory. HK and HL analyzed the data. HL and HK drafted the paper. All authors revised and contributed to the final version of the manuscript.
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cichlids *Geophagus brasiliensis* and *G. Steindachneri*. *Evolution* **45**: 1545-1563.


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Appendices

Table S1. Specimens of Midas cichlid color morphs used for the geometric morphometrics study of body shape.
Total sample size is 1,177.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Species</th>
<th>n gold</th>
<th>n dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asososca Managua</td>
<td><em>A. cf citrinellus</em></td>
<td>51</td>
<td>138</td>
</tr>
<tr>
<td>Apoyeque</td>
<td><em>A. cf citrinellus</em></td>
<td>7</td>
<td>25</td>
</tr>
<tr>
<td>Masaya</td>
<td><em>A. cf citrinellus</em></td>
<td>16</td>
<td>38</td>
</tr>
<tr>
<td>A. cf labiatus</td>
<td>13</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Xiloá</td>
<td><em>A. sagittae</em></td>
<td>6</td>
<td>133</td>
</tr>
<tr>
<td>A. xiloaensis</td>
<td>67</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Managua</td>
<td><em>A. citrinellus</em></td>
<td>60</td>
<td>114</td>
</tr>
<tr>
<td>A. labiatus</td>
<td>4</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Nicaragua</td>
<td><em>A. citrinellus</em></td>
<td>104</td>
<td>120</td>
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<tr>
<td>A. labiatus</td>
<td>114</td>
<td>56</td>
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</table>
Table S2. Specimens of Midas cichlid color morphs used for the geometric morphometrics study of lower pharyngeal shape.
Total sample size is 465. For the complementary caliper measurement approach the sample size was slightly reduced ($n = 456$), because of few damaged and therefore immeasurable jaws.

<table>
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<th>Species</th>
<th>$n_{gold}$</th>
<th>$n_{dark}$</th>
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<td>Asososca</td>
<td>A. cf citrinellus</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Apoyeque</td>
<td>A. cf citrinellus</td>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td>Masaya</td>
<td>A. cf citrinellus</td>
<td>15</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>A. cf labiatus</td>
<td>8</td>
<td>2</td>
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<tr>
<td>Xiloá</td>
<td>A. sagittae</td>
<td>4</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>A. xiloaensis</td>
<td>31</td>
<td>21</td>
</tr>
<tr>
<td>Managua</td>
<td>A. citrinellus</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>A. labiatus</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Nicaragua</td>
<td>A. citrinellus</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>A. labiatus</td>
<td>34</td>
<td>31</td>
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</tbody>
</table>
Table S3. Specimens of Midas cichlid color morphs used for stable isotope analysis. Total sample size is 298.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Species</th>
<th>$n$ gold</th>
<th>$n$ dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asososca</td>
<td><em>A. cf citrinellus</em></td>
<td>36</td>
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<tr>
<td>Managua</td>
<td><em>A. cf citrinellus</em></td>
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<td>11</td>
</tr>
<tr>
<td>Apoyeque</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Masaya</td>
<td><em>A. cf citrinellus</em></td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td><em>A. cf labiatus</em></td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>Xiloá</td>
<td><em>A. sagittae</em></td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td><em>A. xiloaensis</em></td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Managua</td>
<td><em>A. citrinellus</em></td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td><em>A. labiatus</em></td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Nicaragua</td>
<td><em>A. citrinellus</em></td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td><em>A. labiatus</em></td>
<td>14</td>
<td>13</td>
</tr>
</tbody>
</table>
Table S4. Summary data of microsatellite loci.
The table depicts the genetic summary information for each microsatellite locus and refers to the sample of 118 Midas cichlid individuals from Asososca Managua that have been genetically investigated in this study.

<table>
<thead>
<tr>
<th></th>
<th>ABUR 151</th>
<th>ABUR 82</th>
<th>MM</th>
<th>UNH011</th>
<th>UNH0012</th>
<th>M2</th>
<th>M7</th>
<th>TMG7</th>
<th>ABUR45</th>
<th>BUR101</th>
<th>M12</th>
<th>UNH013</th>
<th>UNH002</th>
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</thead>
<tbody>
<tr>
<td>sample size (n)</td>
<td>109</td>
<td>102</td>
<td>110</td>
<td>109</td>
<td>94</td>
<td>113</td>
<td>110</td>
<td>112</td>
<td>113</td>
<td>111</td>
<td>116</td>
<td>112</td>
<td>106</td>
</tr>
<tr>
<td>number of alleles (Ma)</td>
<td>2</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>9</td>
<td>13</td>
<td>14</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>6</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>number of effective alleles (Ne)</td>
<td>1.037</td>
<td>2.094</td>
<td>1.622</td>
<td>1.99</td>
<td>2.22</td>
<td>5.163</td>
<td>9.288</td>
<td>1.671</td>
<td>1.907</td>
<td>2.528</td>
<td>2.214</td>
<td>4.523</td>
<td>2.554</td>
</tr>
<tr>
<td>observed heterozygosity (Ho)</td>
<td>0.018</td>
<td>0.451</td>
<td>0.536</td>
<td>0.55</td>
<td>0.596</td>
<td>0.894</td>
<td>0.882</td>
<td>0.41</td>
<td>0.407</td>
<td>0.586</td>
<td>0.552</td>
<td>0.821</td>
<td>0.566</td>
</tr>
<tr>
<td>gene diversity/respected heterozygosity (He)</td>
<td>0.036</td>
<td>0.522</td>
<td>0.383</td>
<td>0.497</td>
<td>0.55</td>
<td>0.886</td>
<td>0.892</td>
<td>0.401</td>
<td>0.476</td>
<td>0.604</td>
<td>0.548</td>
<td>0.779</td>
<td>0.608</td>
</tr>
<tr>
<td>Inflation index (Fis)</td>
<td>0.491</td>
<td>0.137</td>
<td>0.123</td>
<td>-0.107</td>
<td>-0.084</td>
<td>0.108</td>
<td>0.012</td>
<td>0.045</td>
<td>0.144</td>
<td>0.051</td>
<td>0.006</td>
<td>0.055</td>
<td>0.07</td>
</tr>
</tbody>
</table>
**Table S5. Levene’s tests for homogeneity of variances in stable isotope signatures.**

Shown are variances, test statistics and significance levels of Levene’s tests from between-lake comparisons of Asososca Managua and lakes Apoyo and Xiloá with adjusted (randomly drawn) sample sizes \( n = 74 \) for stable isotopes \( \delta^{15}N \) (A) and \( \delta^{13}C \) (B). In an analogous setup, the respective limnetic species from lakes Apoyo and Xiloá was compared to *A. tolteca* with adjusted sample sizes of \( n = 20 \) (C, D).

<table>
<thead>
<tr>
<th></th>
<th>Levene's test</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>variance</td>
<td>test statistic</td>
<td>( p )-value</td>
</tr>
<tr>
<td>(A) ( \delta^{15}N )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asososca Managua</td>
<td>1.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoyo</td>
<td>0.99</td>
<td>1.34</td>
<td>0.25</td>
</tr>
<tr>
<td>Xiloá</td>
<td>0.90</td>
<td>1.41</td>
<td>0.24</td>
</tr>
<tr>
<td>(B) ( \delta^{13}C )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asososca Managua</td>
<td>3.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoyo</td>
<td>4.39</td>
<td>5.19</td>
<td>0.02</td>
</tr>
<tr>
<td>Xiloá</td>
<td>4.81</td>
<td>12.38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(C) ( \delta^{15}N )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asososca Managua</td>
<td>1.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. zaliogus</em> (Apoyo)</td>
<td>1.06</td>
<td>0.31</td>
<td>0.58</td>
</tr>
<tr>
<td><em>A. sagittae</em> (Xiloá)</td>
<td>0.15</td>
<td>13.18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(D) ( \delta^{13}C )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asososca Managua</td>
<td>4.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. zaliogus</em> (Apoyo)</td>
<td>1.02</td>
<td>12.06</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>A. sagittae</em> (Xiloá)</td>
<td>0.92</td>
<td>12.23</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Table S6. Geometric morphometric data on mouth shape: test of directional asymmetry versus non-directional asymmetry.
Statistical inference of directional asymmetry (effect = side) as well as non-directional asymmetry (i.e. fluctuating asymmetry or antisymmetry) (effect = individual x side) from geometric morphometric data on mouth shape for ca. three months old fish (a) and ca. seven months old fish (b) using Procrustes ANOVA and MANOVA. F - values in boldface denote statistical significance. SS: Sums of squares; MS: mean squares (in dimensionless units of Procrustes distance).

<table>
<thead>
<tr>
<th></th>
<th>Procrustes ANOVA</th>
<th>MANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>effect</td>
<td>SS</td>
</tr>
<tr>
<td>(a) three months</td>
<td>individual</td>
<td>$2.27 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>side</td>
<td>$1.48 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>individual x side</td>
<td>$1.65 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>measurement error</td>
<td>$5.93 \times 10^{-3}$</td>
</tr>
<tr>
<td>(b) seven months</td>
<td>individual</td>
<td>$1.90 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>side</td>
<td>$5.46 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>individual x side</td>
<td>$2.05 \times 10^{-2}$</td>
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<tr>
<td></td>
<td>measurement error</td>
<td>$5.43 \times 10^{-3}$</td>
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Table S7. Lateralized foraging behaviour in adult *Perissodus microlepis* foraging preferences and foraging scores.

<table>
<thead>
<tr>
<th></th>
<th>experimental tank</th>
<th>number of prey fish</th>
<th>left flanks affected</th>
<th>right flanks affected</th>
<th>foraging scores left flank</th>
<th>foraging scores right flank</th>
</tr>
</thead>
<tbody>
<tr>
<td>community</td>
<td>left (6 x L)</td>
<td>18</td>
<td>4</td>
<td>12</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>right (10 x R)</td>
<td>25</td>
<td>20</td>
<td>5</td>
<td>31</td>
<td>7</td>
</tr>
<tr>
<td>pair tanks</td>
<td>disassortative 1 (RL)</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>disassortative 2 (RL)</td>
<td>3</td>
<td>3</td>
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<td>6</td>
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<tr>
<td></td>
<td>disassortative 3 (RL)</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
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<td>3</td>
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<td>4</td>
</tr>
<tr>
<td></td>
<td>disassortative 5 (RL)</td>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>disassortative 6 (RL)</td>
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<td>2</td>
<td>3</td>
<td>2</td>
<td>6</td>
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<td>3</td>
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</tr>
<tr>
<td></td>
<td>assortative left 1 (LL)</td>
<td>6</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>assortative right 1 (RR)</td>
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<td>3</td>
<td>1</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>assortative right 2 (RR)</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>assortative right 3 (RR)</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>assortative right 4 (RR)</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>assortative right 5 (RR)</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>
**Figure S1.**

**Lake Managua, *Amphílopus citrinellus***

**Body shape**

- Scale factor = 5
- Frequency distribution
- Gold: dark = 60.114
- Procrustes distance = 0.01
- Hotel. $T^2 = 67.99$, $p = 0.01$

**LPJ shape**

- Scale factor = 5
- Frequency distribution
- Gold: dark = 24:21
- Procrustes distance = 0.037
- Hotel. $T^2 = 113.38$, $p = 0.014$

**Stable isotopes**

- $d^{15}N$: t = 3.14, $p = 0.005$
- $d^{13}C$: t = 1.06, $p = 0.296$

---

**Lake Managua, *Amphílopus labiatus***

**Body shape**

- Scale factor = 5
- Frequency distribution
- Gold: dark = 4:32
- Procrustes distance = 0.03
- Hotel. $T^2 = 121.04$, $p = 0.954$

**LPJ shape**

- Scale factor = 5
- Frequency distribution
- Gold: dark = 4:13
- Procrustes distance = 0.016
- Hotel. $T^2 = 85.6$, $p = 0.886$

**Stable isotopes**

- $d^{15}N$: t = 0.89, $p = 0.413$
- $d^{13}C$: t = 1.42, $p = 0.187$
Appendices

Figure S1 continued.

Lake Nicaragua, *Amphilopus citrinellus*

**Body shape**

**LPJ shape**

**stable isotopes**

![Graphs showing frequency distribution and discriminant function plots for body and LPJ shapes, along with stable isotope data for gold and dark specimens.](image)

gold:dark = 104:120  
Procrustes distance = 0.012  
Hotel. $T^2 = 189.56, p < 0.0001$

gold:dark = 32:30  
Procrustes distance = 0.006  
Hotel. $T^2 = 24.23, p = 0.817$

gold:dark = 10:11  
d$^{13}$N: $t = 4.06, p = 0.002$

d$^{13}$C: $t = 4.90, p = 0.001$

Lake Nicaragua, *Amphilopus labiatus*

**Body shape**

**LPJ shape**

**stable isotopes**

![Graphs showing frequency distribution and discriminant function plots for body and LPJ shapes, along with stable isotope data for gold and dark specimens.](image)

gold:dark = 114:56  
Procrustes distance = 0.01  
Hotel. $T^2 = 96.25, p < 0.0001$

gold:dark = 34:31  
Procrustes distance = 0.008  
Hotel. $T^2 = 36.99, p = 0.353$

gold:dark = 14:13  
d$^{13}$N: $t = 3.18, p = 0.004$

d$^{13}$C: $t = 1.81, p = 0.082
Figure S1 continued.

Lake Apoyeque, *Amphilophus cf. citrinellus*

**Body shape**

![Body shape diagram]

Gold:dark = 7.25  
Procrustes distance = 0.022  
Hotol. $T^2 = 6002.65, p = 0.304$

**LPJ shape**

![LPJ shape diagram]

Gold:dark = 7.19  
Procrustes distance = 0.019  
Hotol. $T^2 = 368.74, p = 0.328$

**Stable isotopes**

![Stable isotopes diagram]

gold:dark = 8.11  
d$^{15}N$: $t = 2.79$, $p = 0.013$  
d$^{13}C$: $t = 1.42$, $p = 0.175$

---

Lake Xiloá, *Amphilophus sagittae*

**Body shape**

![Body shape diagram]

gold:dark = 6.133  
Procrustes distance = 0.023  
Hotol. $T^2 = 98.22, p = 0.002$

**LPJ shape**

![LPJ shape diagram]

gold:dark = 4.39  
Procrustes distance = 0.025  
Hotol. $T^2 = 49.35, p = 0.414$

**Stable isotopes**

![Stable isotopes diagram]

gold:dark = 2.20  
d$^{15}N$: $t = 0.37$, $p = 0.78$  
d$^{13}C$: $t = -6.86$, $p = 0.039$
Figure S1 continued.

Lake Xiloa, *Amphilopus xiloaensis*

**Body shape**

![Diagram](scale_factor=2)

![Histogram](Frequency vs. Discriminant function)

- gold:dark = 67.77
- Procrustes distance = 0.025
- Hotel. $T^2 = 265.03$, $p < 0.0001$

**LPJ shape**

![Diagram](scale_factor=5)

![Histogram](Frequency vs. Discriminant function)

- gold:dark = 31.21
- Procrustes distance = 0.022
- Hotel. $T^2 = 167.34$, $p < 0.0001$

**Stable isotopes**

- gold:dark = 18.16
- $d_{15}N$: $t = -5.61$, $p < 0.001$
- $d_{13}C$: $t = -2.98$, $p = 0.009$

Lake Asososca Managua, *Amphilopus tolteca*

**Body shape**

![Diagram](scale_factor=5)

![Histogram](Frequency vs. Discriminant function)

- gold:dark = 51.138
- Procrustes distance = 0.011
- Hotel. $T^2 = 118.78$, $p < 0.0001$

**LPJ shape**

![Diagram](scale_factor=5)

![Histogram](Frequency vs. Discriminant function)

- gold:dark = 43.54
- Procrustes distance = 0.012
- Hotel. $T^2 = 80.58$, $p < 0.0001$

**Stable isotopes**

- gold:dark = 36.38
- $d_{15}N$: $t = 1.15$, $p = 0.256$
- $d_{13}C$: $t = 0.33$, $p = 0.742$
Figure S1 continued.

Lake Masaya, *Amphilophus cf. citrinellus*

**Body shape**

![Body shape diagram](image)

- gold:dark = 16.38
- Procrustes distance = 0.013
- Hotel. $T^2 = 116.09$, $p = 0.182$

**LPJ shape**

![LPJ shape diagram](image)

- gold:dark = 15.33
- Procrustes distance = 0.007
- Hotel. $T^2 = 20.85$, $p = 0.931$

**stable isotopes**

- $\delta^{15}N$: $t = -0.2$, $p = 0.844$
- $\delta^{13}C$: $t = 1.81$, $p = 0.089$

Lake Masaya, *Amphilophus cf. labiatus*

**Body shape**

![Body shape diagram](image)

- gold:dark = 13.2
- Procrustes distance = 0.02
- Hotel. $T^2 = 79.69$, $p = 0.766$

**LPJ shape**

![LPJ shape diagram](image)

- gold:dark = 8.2
- Procrustes distance = 0.036
- Hotel. $T^2 = 20.34$, $p = 0.174$

**stable isotopes**

- $\delta^{15}N$: $t = 0.21$, $p = 0.851$
- $\delta^{13}C$: $t = 0.3$, $p = 0.784$

Figure S1. Group-wise ecological differentiation between Midas cichlid color morphs.

From left to right: Discriminant function analyses of body shape, lower pharyngeal jaw morphology and differentiation in stable isotopes $\delta^{15}N$ and $\delta^{13}C$ are depicted along with
sample sizes, test statistics and significance levels for each of the ten investigated populations. The degree of body and pharyngeal jaw shape differentiation is indicated by Procrustes distances. Shape changes are presented as wireframe outline graphs of the landmark configuration and depict the mean shape within each group. Note that for a better visualization of the shape differences scaling was applied as indicated. Significance levels obtained from pair-wise $t$-tests of stable isotope differentiation are indicated as follows: † = $p < 0.1$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. The *gold* morph is generally higher bodied, and has a relatively larger head than the *dark* morph. Lower pharyngeal jaw morphologies were rather robust and molariform in *gold* morphs compared to *dark* morphs. The *gold* morph generally feeds at a lower trophic level than the *dark* morph.
Figure S2. Residual analysis of the stable isotope analysis of Midas cichlid color morphs.

The assumptions of the linear mixed effects model dealing with color morph differentiation in stable isotopes were visually checked. Upper left panel: Plot of residuals vs fitted values to assess possible deviations of the residuals’ mean from zero. Upper right: Q-Q-plot of residuals, to assess whether the residuals conform to normality. Lower left: Plot of residual variance vs. fitted values to evaluate variance homogeneity. Lower right panel: Q-Q-plot of random effects to assess whether random effects are normally distributed after implementation into the model.
Figure S3. Residual analysis of the linear mixed effects model on predation data. The assumptions of the linear mixed effects model were adequately met. The time to attack was modeled as a function of prey fish color, irrespective of the predator and while accounting for attack order. Upper left panel: Plot of residuals vs fitted values to assess possible deviations of the residuals’ mean from zero. Upper right: Q-Q-plot of residuals, to assess whether the residuals conform to normality. Lower left: Plot of residual variance vs. fitted values to evaluate variance homogeneity. Lower right panel: Q-Q-plot of random effects to assess whether random effects are normally distributed after implementation into the model.
Figure S4. Assessing model assumptions: survival analysis.
The Kaplan-Meier survivorship curves are depicted along with the empirical data (black lines). The overall match indicates that the model assumptions were adequately met. The survivor functions (orange lines) show the estimated proportions of survivorship likelihood for each of the four possible prey fish fates; e.g. two color variants (“gold/grey”) and attack order (“first/second”). The black lines indicate the empirical data. As has been found in our previous approach (Figure 12) the difference in attack time (survivorship) between color morphs within each category of capture order was indiscriminant ($p = 0.64$).
Figure S5. Across-predator variance in attack latency.

Although all predators conform to the overall trend, there is variation in attack latency among predators. For each of the twelve predators, the means (dot) along with 95% Credible Interval (bars) of the posterior model distributions are depicted for the estimated time to attack within each category of capture order (“first, second”) of both prey fish color morphs (“gold, grey”). When capture order was corrected for, the time to attack for both color morphs was indiscriminant. Note the differences in scaling of the ordinate axis.
Appendices

Figure S6. PCA of standardized eco-morphological variables.

A) The trait loadings along PCs 1 and 2 are depicted. PCs 1 and 2 encode for 53.42 % of the variation. Variables with the same sign covary in the same direction along PCs 1 and 2, whereas unequal signs indicate correlations in opposite directions. The numbers indicate the strength of correlation along a particular axis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PC 1</th>
<th>PC 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHI</td>
<td>-0.272</td>
<td>0.437</td>
</tr>
<tr>
<td>LPJ weight</td>
<td>-0.455</td>
<td>-0.19</td>
</tr>
<tr>
<td>LPJ width</td>
<td>-0.446</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>LPJ length</td>
<td>-0.393</td>
<td>-0.181</td>
</tr>
<tr>
<td>LPJ depth</td>
<td>-0.405</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>PC1Body</td>
<td>-0.237</td>
<td>0.468</td>
</tr>
<tr>
<td>PC2Body</td>
<td>&lt;0.1</td>
<td>0.499</td>
</tr>
<tr>
<td>PC3Body</td>
<td>-0.133</td>
<td>0.391</td>
</tr>
<tr>
<td>d15N</td>
<td>0.2</td>
<td>0.334</td>
</tr>
<tr>
<td>d13C</td>
<td>-0.293</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

B) The biplot of PCs 1 and 2 indicates strong correlations of the eco-morphological variables. Specimens codings „B“ (high-bodied, benthic) and „E“ (elongated, limnetic) indicate initial field assignments into discrete ecotypes.

Figure S6. PCA of standardized eco-morphological variables.
A) The trait loadings along PCs 1 and 2 are depicted. PCs 1 and 2 encode for 53.42 % of the variation. Variables with the same sign covary in the same direction along PCs 1 and 2, whereas unequal signs indicate correlations in opposite directions. The numbers indicate the strength of correlation along a particular axis. B) The biplot of PCs 1 and 2 indicates strong correlations of the eco-morphological variables. Specimens codings „B“ (high-bodied, benthic) and „E“ (elongated, limnetic) indicate initial field assignments into discrete ecotypes.
Figure S7. Deviation from normality in ecologically relevant traits.
Shown are normal probability plots with cumulative proportions of observed versus expected proportions in comparison to a single normal distribution (red line) for LPJ variables and stable isotopes $\delta^{15}$N and $\delta^{13}$C.
Figure S8. Fitting two normal distributions to the data sets used in this study. The parametric outcome of this EM (expectation–maximization) algorithm-based approach was used for the bootstrap test to infer whether the trait distribution most likely consisted of one or two components.
Figure S9. Description of landmark (LM) configuration on the upper jaw outline of laboratory reared juvenile fish.
LM 1 was defined at the tip of the snout. LM 2 and 3 were defined at the two frontal eye sockets. LM 4 was then identified at the mid-point between LM 2 and 3. Auxiliary points $a$ and $a'$ indicate the mid-point between LM 1 and 2 and LM 1 and 3. LM 5 is the point where a line from LM 3 through $a$ intersects the upper jaw margin. LM 6 is the point where a line from LM 2 through $a'$ intersects the upper jaw margin. Auxiliary points $b$ and $b'$ indicate the mid-point between LM 1 and 5 and LM 1 and 6. LM 7 is the point where a line from LM 3 through $b$ intersects the upper jaw margin. LM 8 is the point where a line from LM 2 through $b'$ intersects the upper jaw margin. Auxiliary points $c$ and $c'$ indicate the mid-point between LM 1 and 7 and LM 1 and 8. LM 9 is the point where a line from LM 3 through $c$ intersects the upper jaw margin. LM 10 is the point where a line from LM 2 through $c'$ intersects the upper jaw margin.