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Parental care in the Cuatro Ciénegas cichlid, *Herichthys minckleyi* (Teleostei:
Cichlidae)

Short title: **Parental care in the Cuatro Ciénegas cichlid**

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Word count: Title: 11; Abstract: 196 (200 max.); Text: 15,918 (Abstract - Figure legends)

Tables: 4; Figures: 9; Appendix: 1

Abstract Behavioral studies have often examined parental care by measuring phenotypic plasticity of behavior within a species. Phylogenetic studies have compared parental care among species, but only at broad categories (e.g., care vs. no care). Here we provide a detailed account that integrates phylogenetic analysis with quantitative behavioral data to better understand parental care behavior in the Cuatro Ciénegas cichlid, *Herichthys minckleyi*. We found that *H. minckleyi* occurs in a clade of sexually monochromatic or weakly dichromatic monogamous species, but that male and female *H. minckleyi* have dramatically different reproductive coloration, likely as a result of sexual selection. Furthermore, we found that males are polygynous; large males guard large territories and smaller males may attempt alternative mating tactics (sneaking). Finally, compared to the closely related monogamous Rio Grande cichlid, *H. cyanoguttatus*, males of *H. minckleyi* were present at their nests less often and performed lower rates of aggressive offspring defense and females compensated for the absence of their mates by performing higher levels of offspring defense. Body color, mating system, and parental care in *H. minckleyi* appear to have evolved after it colonized Cuatro Ciénegas, and are likely a result of evolution in an isolated, stable environment.

Keywords aggression · Cuatro Ciénegas · *Herichthys* · mating systems · polygyny · sexual selection

Introduction

Parental care is any form of parental behavior that increases the fitness of the offspring (Gonzalez-Voyer & Kolm, 2010; Royle et al., 2012). Current understanding of parental care evolution is that of a complex process in which several factors interact to determine if males and/or females provide care. Factors such as adult sex ratio (ASR), distribution of resources and competitors, and faster rate of gamete production in males influence the operational sex ratio (OSR): the ratio of reproductively capable males to reproductively capable females (Baylis, 1981; Kvarnemo & Ahnesjö, 1996). A skewed OSR may result in competition for mates and non-random variance in mating success among males or females, i.e., sexual selection (Cunningham & Birkhead, 1998; Kokko et al., 2012). Skewed OSR and sexual selection can influence the time, place, and partner(s) during mating, i.e., mating systems (Emlen & Oring, 1977; Davies, 1989; Gonzalez-Voyer et al., 2008). Parental care has long been known to be closely associated with mating system (Barlow, 1991; Reynolds, 1996), and recent phylogenetic studies have suggested that they often evolve together and may influence each other (Ah-King et al., 2005; Thomas & Székely, 2005; Olson et al., 2009). A need for a high level of parental care to ensure offspring survival might select monogamous mating behavior (Barlow, 1974, 1991; Thomas & Székely, 2005). In addition, drivers of mating system such as OSR and sexual selection could also influence parental care (Kokko & Jennions, 2008). However, changes in average rates of parental care behavior over evolutionary time are not well understood.

Many ethological studies have tested parental care theories by measuring

phenotypically plastic changes in behavior in individuals of a single species after manipulating the availability of (potential) mates or resources (Mock & Fugioka, 1990; Westneat & Sargent, 1996). However, Kokko & Jennions (2008) pointed out that tests of phenotypic plasticity do not necessarily indicate how care evolves. One reason is because such tests do not address the feedback effects that occur over longer periods (Kokko & Jennions, 2008, 2012; Alonzo, 2010). For example, manipulation of ASR can cause male desertion (Keenleyside, 1983), but in the long term male desertion will alter OSR. One way to examine long-term changes in parental care over evolutionary time is to compare behavior between species. The value of comparative studies in understanding evolution of behavior was apparent to early biologists including Darwin, Lorenz, and Tinbergen (reviewed by Price et al., 2011) and recent studies have specifically compared parental care behavior between species (Lehtonen et al., 2011a; van Dijk et al., 2010). While such studies may be able to conclude that evolution is responsible for differences observed between species, without knowledge of the phylogenetic relationships among the species such studies are not able to make conclusions about the direction of the evolutionary change or the circumstances surrounding it.

Phylogenetic analysis produces parsimonious hypotheses about the past, making it possible to reconstruct the most likely ancestral state of a trait and identify evolutionary transitions (Fink, 1982; Martins, 1996; Chakrabarty, 2011a). Gittleman (1989) described several different applications for which phylogenies and ancestral state reconstruction might be used to study evolution of behavior. One application is to search for parallel evolutionary transitions in different lineages to identify evolutionary patterns or trends,

which typically requires behavior to be represented in low-resolution categories, e.g., “parental care” vs. “no parental care” (Mank et al., 2005; Olson et al., 2009). Another application is to identify the lineage in which a particular evolutionary transition occurred to examine subtle changes in the behavior and the circumstances surrounding it. Both of these two approaches were previously hindered by statistical difficulties in ancestral state reconstruction (Gittleman, 1989; Losos, 1999). Recent conceptual/statistical advances have allowed several studies of evolutionary trends in broad categories of behavior (e.g., Mank et al., 2005; Olson et al., 2009). However, the use of phylogeny to provide a context in which to understand behavior in a single focal lineage has remained largely unexplored; indeed, Price et al. (2011) noted a decline in the number of studies containing phylogenetic information published in animal behavior journals in recent years.

Cichlidae is a species-rich family of freshwater fishes (Nelson, 2006) that includes diverse mating systems and parental care forms such as substrate spawning and mouthbrooding, and uniparental and biparental care (Barlow, 2000). The cichlid tribe Heroini consists of approximately 150 species ranging from northern South America to the USA. Most species occur in Central America but one species, the Rio Grande cichlid, *Herichthys cyanoguttatus* (Baird & Girard, 1854), reaches as far north as Texas (Reis et al., 2003). Monogamous biparental care has been suggested to be the ancestral form of parental care in heroines (Goodwin et al., 1998). Unlike most fish species, heroines are well known for forming male-female pair bonds and providing biparental care to their offspring, and for decades they have served as model species for studying monogamy

(Baerends & Baerends van Roon, 1950; Barlow 2000). For example, in *H. cyanoguttatus*, reproduction involves a male and a female forming a bond, mating, and then cooperating to defend their offspring (Buchanan, 1971; Itzkowitz & Nyby, 1982). Furthermore, in heroine cichlids males and females typically exhibit the same color patterns – a trait that is often associated with monogamous mating systems and indicates a lack of strong sexual selection in body color (Badyaev & Hill, 2003) (although other traits, such as body size, may show evidence of sexual selection). Both the male and the female may change color when breeding, but in most species they both undergo the same change in color pattern (although one sex sometimes exhibits aspects of the pattern more intensely than the other; Baerends & Baerends van Roon, 1950; Barlow, 1974).

The Cuatro Ciénegas cichlid, *H. minckleyi* (Kornfield & Taylor, 1983), is a heroine cichlid that, remarkably, appears to mate polygynously (Kornfield et al., 1982), suggesting that polygyny evolved at some time during the diversification of heroine cichlids. *Herichthys minckleyi* also exhibits a trophic polymorphism that has received significant attention (Kornfield & Koehn, 1975; Sage & Selander, 1975; Kornfield & Taylor, 1983; Liem & Kaufman, 1984; Swanson et al., 2003; Trapani 2003a,b,c; Cohen et al., 2005; Hulsey et al., 2006). Indication that this species might not be monogamous was suggested by observations among different trophic morphs that revealed lone females guarding offspring and some males guarding two brood sites simultaneously (Kornfield et al., 1982). Furthermore, *H. minckleyi* has a distinctly sexually dichromatic color pattern, a trait often associated with polygynous mating systems (Cunningham & Birkhead, 1998). These compelling characteristics of mating behavior and body color in

H. minckleyi recently stimulated an analysis of neural gene expression in relation to mating system (Oldfield et al., 2013).

In the current study we report observations regarding body color and the possibility of sexual selection, mating system including alternative mating tactics, and patterns of parental care behavior in *H. minckleyi*. We begin with a phylogenetic analysis and examine the distribution of body color patterns within the genus *Herichthys* and the closely related genus *Nosferatu* (de la Maza-Benignos et al., 2014). Next, we present our observations of the mating system of *H. minckleyi* and of sneaking behavior performed as an alternative male mating tactic. Finally, we investigated parental care behavior by conducting field observations of reproductively active adults of *H. minckleyi* and *H. cyanoguttatus* and quantifying rates of behavior with the expectation that in the polygynous mating system of *H. minckleyi* males would perform less parental care compared to the well studied, monogamous *H. cyanoguttatus*. Specifically, we expected that in *H. cyanoguttatus* (which exhibits monogamy, the putatively ancestral state), we would observe no females guarding offspring without a male partner present, as was observed by Itzkowitz & Nyby (1982). However, we predicted (1) that in *H. minckleyi* (which exhibits polygyny, the putatively derived state), males would accompany females and offspring less often than in *H. cyanoguttatus*. Furthermore, we predicted (2) that, when present, male *H. minckleyi* would perform lower quantitative rates of parental care behavior (*sensu* Keenleyside, 1983; Itzkowitz, 1984; Balshine-Earn, 1995; Grüter & Taborsky, 2005) in the form of brood defense and also maintain a greater distance from the brood site than would male *H. cyanoguttatus*. Finally, because of the high level of

sexual conflict associated with polygynous mating systems (Westneat & Sargent, 1996), we predicted (3) that female *H. minckleyi* might compensate for a reduction in male brood defense by increasing their own offspring-defense behavior and by remaining closer to their offspring than female *H. cyanoguttatus* (Itzkowitz, 1984).

Methods

Subjects

Herichthys minckleyi is one of seven described species in the monophyletic genus *Herichthys* and is endemic to the isolated valley of Cuatro Ciénegas in the Chihuahuan Desert of northern Mexico (Miller et al., 2005; Hulsey et al., 2010; McMahan et al., 2010; de la Maza-Benignos et al., 2014). Cuatro Ciénegas is characterized by a complex system of geothermal springs and pools and a large proportion of species there are endemic (Minckley, 1969; Sage & Selander, 1975). In this environment, *H. minckleyi* evolved a trophic polymorphism in which some individuals with papilliform pharyngeal teeth specialize on plant material but others with molariform pharyngeal teeth may exploit the extremely hard-shelled snails that are endemic to the region (Kornfield & Koehn, 1975; Sage & Selander, 1975; Kornfield & Taylor, 1983; Liem & Kaufman, 1984; Swanson et al., 2003; Trapani 2003a,b,c; Cohen et al., 2005; Hulsey et al., 2006). In addition, observations of lone females guarding offspring and males guarding two brood sites simultaneously suggested that the species has a polygynous mating system (Kornfield et al., 1982). Furthermore, *H. minckleyi* have a sexually dichromatic color pattern; when not breeding, both female and male *H. minckleyi* have a flat grey

background color overlaid with small iridescent blue-green specks. When breeding, females turn white and males may turn completely black (Kornfield & Taylor, 1983). Sexual dichromatism is often a result of sexual selection, which is typically stronger in polygynous mating systems than in monogamous mating systems (Cunningham & Birkhead, 1998).

We also examined *Herichthys cyanoguttatus*, an ecologically generalist heroine cichlid (Smith et al., 2008) that has a relatively wide native distribution in drainages of the Gulf Coast of northern Mexico and southern Texas (Brown, 1953) and is closely related to *H. minckleyi* (Hulseay et al., 2010; McMahan et al., 2010, de la Maza-Benignos et al. 2014). This species mates in a manner typical of substrate spawning monogamous cichlids (Baerends & Baerends van Roon, 1950; Buchanan, 1971; Itzkowitz & Nyby, 1982). Furthermore, *H. cyanoguttatus* is sexually monochromatic (Buchanan, 1971), which is often associated with monogamy (Cunningham & Birkhead, 1998; Badyaev & Hill, 2003). When not breeding, both female and male *H. cyanoguttatus* have a flat grey background color overlaid with small iridescent blue-green spots. When breeding, the anterior half of both females and males turns white and the posterior half turns black, although the white in males is often less intense than in females (Buchanan, 1971).

General observations

The general observations reported here stem from many years of both systematically and casually observing *H. minckleyi* in Cuatro Ciénegas and in captivity. In several contexts, we compare our observations of *H. minckleyi* with similar observations of *H.*

cyanoguttatus. Because these general observations are the cumulative result of collective observations of multiple authors over many years, we do not report date of data collection, identity of the observer, or sample size; the sample sizes are unknown but in most cases are based on observations of at least dozens of individuals.

RGO, KM, BOS, and DAH observed *H. minckleyi* in Cuatro Ciénegas, Mexico (26.983°N, 102.050°W) in various pools and one river, which were characterized by clear water, stromatolite formations, vegetation, and travertine, gravel, and detritus substrates (Swanson et al., 2003; Cohen et al., 2005). We have no detailed information on spawning seasonality in *H. minckleyi*, but opportunistic observations suggest that spawning occurs year-round. RGO, KM, DAH, and HAH observed *H. cyanoguttatus* near The University of Texas at Austin campus in Shoal Creek in Austin, Texas (30.283085° N, 97.751727° W) and on the campus of Texas State University in the wetland area of Spring Lake in San Marcos, Texas (29.89096° N, 97.933466° W), which is the origin of the San Marcos River. (Note that *H. cyanoguttatus* is introduced at both of these sites [Brown, 1953], but the sites are close to its natural range. Furthermore, the San Marcos site was previously used by Buchanan [1971] and by Itkowitz & Nyby [1982], facilitating comparisons between our data and theirs). Observations in Shoal Creek were made in clear, slow flowing water ranging from approximately 1 to 10 m across and from a few cm to 0.5 m deep over rock substrate. Observations in Spring Lake were made in water approximately 0.5 m deep over silty substrate and with a high density of vegetation. In the San Marcos River, territoriality and pair formation begin in mid-February; spawning begins in late March, peaks in mid-late April, and ends in early September (Buchanan, 1971).

234 Observations were made in a manner similar to those of Itzkowitz and Nyby
235 (1982). An observer stood at the water's edge (on a floating boardwalk at Spring Lake)
236 and recorded observations on a clipboard. At some sites in Cuatro Ciénegas (e.g., Rio
237 Mesquites), water was deeper and observers entered the water with snorkeling gear and
238 an underwater clipboard to record observations. Each reproductively active fish was
239 easily identified as either female or male due to the larger body size of adult males than
240 adult females, as is typical in heroine cichlids (McKaye, 1986). There was no indication
241 that the presence of the observer affected the social behavior of the fish at any of the
242 locations after a short acclimation period.

243 Observations of captive specimens of live *H. minckleyi* stem from fish that were
244 collected in February 1993 from Posos de la Becerra, Cuatro Ciénegas (the type locality
245 of the species; Kornfield & Taylor, 1983) by Gary Kratochvil and Ad Konings (Konings,
246 1994) and bred in captivity. Nine live first-generation offspring were accessioned at
247 Instituto de Biología at the Universidad Nacional Autónoma de México (UNAM) and
248 given on loan to DAH at Texas Natural History Collections (TNHC). These specimens
249 founded a population maintained at TNHC from 1995 to present. The stock was
250 maintained first in aquaria, but starting 24 June 2000 some fish were moved to two
251 interconnected, concrete-lined, well-fed outdoor ponds (24 x 24 m; 2 m deep) at the
252 University of Texas at Austin's Pickle Research campus (30.388420° N, 97.724900° W)
253 where the population grew to > 1000 individuals. The ponds contained nothing but water,
254 fish, a detritus substrate, and eventually dense stands of aquatic plants (*Typha* sp.
255 [Typhaceae], *Potamogeton* sp. [Potamogetonaceae], *Chara* sp. [Characeae]).

Observations in the ponds were performed as described for wild fish. In 2013 the ponds were converted to municipal chlorinated water and the population in the ponds was returned to aquaria. RGO took some offspring on 15 August 2008 and 8 August 2013 and maintained them to the present in aquaria in Cleveland, Ohio. Observations of captive *H. cyanoguttatus* are based on live fish collected in Shoal Creek on 14 August 2008 and 9 August 2013 (Texas Freshwater Fishing License #958060054579) and maintained in aquaria in Cleveland, Ohio. Observations of live, captive fish in the artificial ponds and in aquaria were made by RGO, KM, DAH, and HAH.

Body color

To serve as a framework for analysis of character state evolution (Chakrabarty, 2011b) of body color, PC performed a phylogenetic analysis of nine *Herichthys* and *Nosferatu* species and one outgroup cichlid (*Thorichthys meeki* Brind, 1918) using published sequences (GenBank) for cytochrome b and S7 (Table 1). We partitioned our analyses by gene and constructed a phylogeny using the program MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003). The branch length prior was set to unconstrained with an exponential distribution with the parameter set to 100.0 to avoid artificially long branches (Brown et al., 2010). All priors were unlinked and we ran this analysis for 10 million generations and sampled every 1000. We performed diagnostic tests to evaluate mixing and convergence of MCMC chains and the burn-in was determined from visual inspection of the likelihood plots in the program Tracer v 1.5 (Rambaut & Drummond, 2009).

278 We then reconstructed the evolution of body color in *Herichthys* and *Nosferatu*
279 (Figure 1). We gathered available information from published literature that indicated
280 whether each species was sexually monochromatic or dichromatic (Table 1).
281 Reproductive body coloration was coded as follows: 0 = monochromatic or weakly
282 sexually dichromatic, 1 = strongly sexually dichromatic. We defined weak sexual
283 dichromatism as characterizing species in which the female and the male exhibit the same
284 color pattern, but in which either the male or female may display some aspect of the
285 pattern more intensely than the other sex. This contrasts with what we defined as strong
286 sexual dichromatism, the expression of distinctly different color patterns in males and
287 females. We then performed an ancestral state reconstruction of body color using
288 Mesquite 2.7, which provides quantitative maximum likelihood values that indicate a
289 degree of confidence for the reconstructed state and allow an inference of where each
290 evolutionary transition occurred in the phylogeny. The Mk1 model (Lewis, 2001) was
291 used to identify the state at each node that maximizes the probability of the states
292 observed in the terminal taxa under a likelihood framework.

293 To determine if all-black body color is associated with either feeding behavior or
294 with pharyngeal jaw morphology in *H. minckleyi*, MXN examined 21 one-hour
295 underwater videos filmed by BOS in El Mojorral Oeste (n=20) and Escobedo (n=1) and
296 described in detail previously (Swanson et al., 2005). In short, 70 *H. minckleyi* were
297 captured in 2001, jaw morphology identified as either molariform or papilliform, tagged,
298 and released. Thirteen of the videos were taken 7-11 January 2001 and the other 8 were
299 taken in July of the same year. MXN recorded body color, tag color, tag number, time

observed, and feeding behavior during observations of videos. He was blinded to the meaning of the tag color, which corresponded to jaw morphology. Multiple sightings of the same individual were consolidated into one data point: Any fish observed one time with all-black body color was considered to be capable of expressing that color pattern and any fish with at least one feeding entry was considered feeding. Fishers Exact Test was used to determine relationships between body color and jaw morphology, body color and feeding, and jaw morphology and feeding. Cochran-Mantel-Haenszel test was used to analyze the three variables together simultaneously to determine if they were independent of each other.

Mating system

As we did for body color, for mating system we gathered (from published literature) information on nine species of *Herichthys* and *Nosferatu*, and on *Thorichthys meeki*. Compared to the rich literature on body color, there was relatively limited information available on mating system in *Herichthys* and *Nosferatu*, so we did not perform a phylogenetic analysis and ancestral reconstruction (see Table 1).

We characterized mating system in *H. minckleyi* by observing them in Cuatro Ciénegas and in the artificial ponds in Austin, Texas and compared those observations to observations of *H. cyanoguttatus* in Shoal Creek and Spring Lake. In Cuatro Ciénegas, we made general observations of *H. minckleyi*, as described above, at the sites Tortuguera (Visitor Center), uppermost part of El Mojarral Este, Rio Mesquites, Churince, Los Hundidos, Los Gatos, and Escobedo. In the artificial pools in Texas, RGO entered the

water with snorkeling gear and an underwater clipboard and recorded the general behavior of reproductively active males and females on a total of 10 days between 17 June and 13 July, 2010. Once a large, reproductive female or male was located, it was focally observed for up to 15 min. and its use of space in the pool recorded on a hand drawn map. Territorial male *H. minckleyi* were individually recognizable and could be followed from one observation day to the next. *Herichthys cyanoguttatus* was observed in Shoal Creek and in Spring Lake as described above and the positions of reproductively active males and females were recorded on a hand drawn map. We combined the information from all the observations into composite maps to show the typical densities and territory sizes of reproductively active individuals of each species.

Additionally, we made observations of breeding individuals in aquaria in Austin, Texas and in Cleveland, OH. Alternative male mating behavior (sneaking) was observed in one particular large aquarium (1131 l: 244 x 61 x 76 cm; set up with natural gravel, rocks, and wood, and maintained at 28° C). The sneaking behavior was observed in one individual in a group of adult *H. minckleyi* (9 males and females of various sizes and ages) that was moved to the aquarium on 18 September 2012 and observed daily. General information about the aquarium is available elsewhere (Oldfield, 2009).

Parental care

RGO and KM recorded quantitative behavioral data of *H. minckleyi* in Cuatro Ciénegas on 7-11 August 2008 at the following sites: Tortuguera (Visitor Center) (n = 7), uppermost part of El Mojarral Este (n = 4), Rio Mesquites (n = 4), Churince (n = 2), Los

Hundidos (n = 5), Los Gatos (n = 2), Escobedo (n = 2). Data from all sites were combined for statistical analysis to account for phenotypically plastic variation in behavior that might occur as a response to local ecological conditions at each site. At some sites (e.g., Rio Mesquites), water was deeper and observers entered the water with snorkeling gear and an underwater clipboard to record observations. RGO and KM recorded behavior of *H. cyanoguttatus* in Shoal Creek between 27 June and 4 August, 2008, and in Spring Lake on 13 August, 2008. For *H. cyanoguttatus*, data from both sites (Shoal Creek: n = 10, Spring Lake: n = 5) were combined for analysis to account for phenotypically plastic variation in behavior that might occur in response to local ecological conditions at each site. On 15 August 2008, RGO and KM collected data from *H. minckleyi* in the artificial ponds in Austin, Texas to compare with the wild fish.

Data collection began when an individual *H. minckleyi* or *H. cyanoguttatus* was observed guarding a nest or offspring. Behavior of the guarding female and its male mate, if present, were recorded for 15 min. Every 10 s the observer estimated and recorded the distance to the nearest dm of each parent from the brood-site or free-swimming offspring (depending on the stage of the reproductive cycle). For each fish, all of the distances recorded were averaged to one value. Before observations began, distances were calibrated to environmental features at each site by placing a meter stick into the water. Direct parental care in the form of fanning the eggs was recorded initially, but 3-D structure made it difficult to observe in some replicates so it was not considered further. Indirect care of offspring in the form of bouts of aggressive behavior of focal individuals toward potential brood site competitors and brood predators (conspecifics and other

species) was recorded and counted as either attacks or displays according to Oldfield & Hofmann (2011). Under these conditions each "attack" began with a rapid "charge", some of which ended in a "bite" or "chase". Regardless of how it ended, each "charge" was considered one "attack". Each "display" began with a relatively slower "approach" that typically ended in a slight elevation of the median fins and opercula. Each fish was observed only one time.

To analyze male presence at a nest, the numbers of reproductively active females observed with a male mate and the numbers observed without a male mate during the 15 min. observation period were compared between the two species. This was done while controlling for stage in the reproductive cycle (early stage: offspring not yet produced, eggs, or wrigglers; late stage: free-swimming fry) with a Mantel-Haenszel-Cochran test (McDonald, 2009). In addition, Fisher's exact probability test was used to compare male presence in *H. minckleyi* during the early stages of reproduction vs. the late stages of reproduction. Data collected from the fish in the artificial ponds have been reported previously (Oldfield et al., 2013), but are shown again here for comparison with the wild fish.

To compare parental care behavior of *H. minckleyi* (using only data from Cuatro Ciénegas) and *H. cyanoguttatus*, we analyzed the dependent variables attacks, displays, and distance to nest/offspring. Independent variables included species, sex, and reproductive stage. We only included data from replicates for which both the male and female were observed for at least 10 min. of the 15 min. observation period; i.e., all 15 of the *H. cyanoguttatus* pairs, and a total of 10 *H. minckleyi* pairs from sites Tortuguera (n =

3), El Mojarral Este (n = 1), Rio Mesquites (n = 1), Los Hundidos (n = 2), Los Gatos (n = 2), Escobedo (n = 1): 6 pairs in the early stages of reproduction, 4 pairs at the later stage of reproduction. If a male travelled out of sight then a value of 5 m was entered for distance to the nest/offspring. This distance was much farther than the mean distances observed for females and males of both species, and was typically the maximum distance an observer could reliably estimate. Fish that travelled this far typically appeared not to be engaged in any type of parental care.

First, RGO performed a multivariate test of significance (MANOVA) to determine if overall there were any significant effects in the data set. When this test revealed statistically significant effects, he performed univariate general linear models based on a normal distribution (ANOVA). Additionally, for our dependent variables that consisted of counts of behaviours (attacks and displays) he also used a generalized linear model based on a Poisson distribution (Poisson GLM), because a Poisson distribution better approximates the natural distribution of count data than does a normal distribution (O'Hara & Kotze, 2010). When the univariate tests revealed statistically significant differences then RGO performed simple-effects tests (t-tests) to determine specifically which cells of data differed. The MANOVA, the ANOVAs, and the t-tests were performed using Minitab 16. The Poisson GLM was performed in SPSS 21.

For the MANOVA, the ANOVAs, and the simple-effects tests, in order to include the five fish that were observed for a substantial amount of time (more than 10 min.) but less than the entire 15 min. observation period (two *H. minckleyi* males, one *H. minckleyi* female that was attended by a male, and two *H. minckleyi* females that were not attended

by a male), attacks and displays were converted to frequency per minute for all individuals in the analysis. All data were $[(\log_{10}X)+1]$ transformed to improve normality of distribution (as indicated by Kolmogorov-Smirnov tests) and equality of variance (as indicated by Levene's tests) before analysis. For the MANOVA, in order to maximize power and avoid excessive colinearity, frequencies of aggressive displays and attacks were combined into the dependent variable total aggression. Total aggression and distance to the nest/offspring for both males and females of both species were analysed. After the MANOVA revealed significant effects, ANOVAs were performed for attacks and displays separately, and for distance to nest/offspring.

For the Poisson GLM, RGO analyzed numbers of attacks and displays using a loglinear link function. If data were over dispersed for a Poisson distribution a negative binomial distribution was used instead. Because Poisson GLM's are a relatively new trend in biology, and because the substitution of a Poisson distribution with a negative binomial distribution provides an added degree of uncertainty, we show the output of both the ANOVAs and the Poisson GLMs. To include fish that were observed for more than 10 min. but less than the total 15 min. observation period, instead of converting raw counts to frequencies per minute as in the ANOVAs, RGO used the raw values and included the amount of time observed for each fish in the model as an offset variable.

The effects of male presence/absence on female behavior could not be included as an independent variable in the MANOVA described above due to its unbalanced distribution in the data set (*H. cyanoguttatus* females were always observed with a male present, but *H. minckleyi* females were sometimes observed without a male present).

Consequently, the effect of male presence was analyzed by separate two-way ANOVAs and Poisson GLMs that compared attended ($n = 6$) vs. unattended ($n = 6$) female *H. minckleyi* in the early stages of reproduction with attended ($n = 4$) vs. unattended ($n = 8$) female *H. minckleyi* at the later stage of reproduction. Two of the total 26 female *H. minckleyi* were not included because they were observed either with or without a male present for less than 10 minutes of the total 15 minute observation period.

After an interaction was found to be significant by the above-described univariate ANOVAs or Poisson GLMs then simple-effects tests (two-tailed t-tests with Welch's correction) were performed to compare each cell with each other cell to identify differences. \log_{10} transformations could not rectify severe departures from normality for the distance to offspring for both early stage unattended female *H. minckleyi* and early stage attended female *H. minckleyi* ($P < 0.010$ for each cell), so to test for simple effects RGO compared those cells using Minitab software's version of the non-parametric Mann-Whitney U test: the Mann-Whitney W test, instead of t-tests. For the t-tests and the Mann-Whitney W test, RGO adjusted the α values to account for multiple comparisons using the method of Benjamini & Hochberg (1995). Therefore, we report the exact p-value produced for each correlation analysis and indicate whether it fell below its adjusted α .

Ethical note

Research was carried out under The University of Texas at Austin IACUC Protocol

#08013002. No animals were harmed. Observations were made carefully so as not to disturb the natural behavior of the animals. None of our general observations or data-collecting observations resulted in a negative response by the animals. No parents fled at our approach and no offspring were consumed by the parents or by other fishes as a result of our observations.

Results

Body color

Sexual monochromatism / weak sexual dichromatism was ubiquitous throughout the genus *Herichthys*, except for *H. minckleyi*, which was the only species characterized by strong sexual dichromatism. Our ancestral state reconstruction found the most recent ancestor of *H. minckleyi* to be sexually monochromatic / weakly sexually dichromatic (91% probability). Therefore, strong sexual dichromatism evolved in the *H. minckleyi* lineage after its divergence from the ancestors of other extant species.

Body color in *H. minckleyi* was variable: we found that the background color in non-breeding individuals ranged from light gray (Fig. 2a) or tan to yellow, green, or dark green, although the yellow and green varieties (Fig. 2b) did not develop a high level of intensity in aquaria. We also observed oligomelanic individuals in the wild (Fig. 2c) and we saw an aquarium specimen transform from the normal gray coloration to an albino condition, which coincided with and may have been related to an illness (Fig. 2d).

In wild and captive individuals of *H. minckleyi*, both males and females underwent a color change upon breeding that was different than that typically observed in

other *Herichthys* species, such as *H. cyanoguttatus* (Fig. 3a, b). Females of *H. minckleyi* typically changed to snowy white color with distinctly contrasting black markings along the flanks, and males to solid dark green or black with the body and fins peppered with pale bluish spots, but we observed other color patterns as well. We saw some breeding females and males that exhibited the half-black, half-white color pattern typical in other *Herichthys* species (Fig. 3c). As in other *Herichthys* species, each breeding color pattern was often partially expressed, and males often exhibited less intense breeding color than females. Intermediate color displays were especially common in captive individuals (Fig. 3d). Additionally, we saw some breeding males that exhibited a light background with contrasting black spots, like typical breeding females of *H. minckleyi* (Fig. 3e).

In males, the all-black color pattern seemed to function in competitive male-male interactions and the light color seemed to communicate pair bonding (Fig. 3). In Cuatro Ciénegas, all-black males were observed engaged in ritualized aggressive lateral displays with one another (Fig. 3f). In one instance, KM observed three all-black males competing over an insect prey. One male seized the prey and then fled and turned green as other individuals attempted to steal it (Fig. 3g, h). RGO observed an all-black male swimming a long distance and interacting with other individuals, seemingly unattached to any particular nest or territory. Eventually it approached a white, brooding female. The female directed pair-bond behavior toward the male (an affiliative open-mouth touch, *sensu* Oldfield and Hofmann 2011) and the male immediately transformed to the white color pattern (Fig. 3e).

We also observed individuals of *H. minckleyi* that were not large males exhibiting

all-black body color. We found that all-black body color was not associated with feeding behavior or with jaw morphology type (Fig. 4). Forty seven tagged fish were observed in the videos. Of these 47, 25 were found to be feeding and 22 were found not to be feeding. Twenty nine fish were papilliform morphs, while 18 were molariform. Fifteen of the fish were black, 32 were gray/green, and none were white. Fish that were feeding were not more likely to be black than not black ($p = 0.505$). Fish of each jaw morphology were equally likely to be observed feeding ($p = 0.771$). Fish of each jaw morphology were equally likely to exhibit black body color ($p = 0.111$). The Cochran-Mantel-Haenszel test indicated that none of the variables were related to any of the other variables (chi-square = 0.713; $p = 0.398$). This is consistent with our aquarium observations: in some broods of juveniles, particular fish sometimes exhibited all-black color when neither feeding nor behaving territorially or reproducing (in other broods all-black coloration was never seen). In contrast, in captive pond and aquarium fish, territorial adult males rarely exhibited all-black body color.

Mating system

Reproductively active male *H. minckleyi* maintained large territories several meters in diameter. In Cuatro Ciénegas, large, all-black males guarded large territories that included ≥ 1 female (Fig. 5a). In each artificial pond in Austin, Texas, four large male *H. minckleyi* each maintained a large territory (Fig. 5b). Although each pond contained several hundred individuals, no other males held territories. Each territory contained one or more nests, which were deep cylindrical holes in the vegetation and detritus substrate,

and one or more brooding females were observed in each male territory. Brooding females were reclusive until their offspring became free-swimming, at which time they were seen guarding their offspring within the same male's territory day after day. In the artificial ponds non-territorial males were not reproductively successful – in one case a small, non-territorial male and female attempted to spawn but were mobbed by dozens of conspecifics that rapidly consumed their eggs. At one site in Cuatro Ciénegas, Los Hundidos, RGO observed three lone, small females exhibiting the white color pattern and defending a row of nestless territories along the shore. In contrast, reproductively active male and female *H. cyanoguttatus* formed pairs that guarded small territories approximately 1 m in diameter (Fig. 5c). Although we did not quantify mate-affiliation behavior typical of paired cichlids (Oldfield & Hofmann, 2011), it seemed to be performed by both males and females of *H. cyanoguttatus* pairs, and was occasionally performed by females of *H. minckleyi*, but was never performed by territorial males of *H. minckleyi*, although males occasionally came into close vicinity of brooding females.

Alternative male mating behavior was observed in the group of 9 adults held in the 1131 L aquarium (Fig. 6a). One male was larger than all the other fish, was dominant over the other fish, and regularly bred with one particular female (which was larger than and dominant over all other females). We never observed polygynous mating in this male, although it was regularly courted by two other large adult females. Although the male attained dark color over the full body at times, it never reached the deep black observed in wild males. On the morning of 23 September 2012 around 10:00 A.M. the large, mated pair began spawning. A much smaller fish hovered high above the spawning

site, behind a log (Fig. 6b). Despite its small body size, it had a male-shaped genital papilla (Barlow, 1976) that was swollen to a remarkably large size (Fig. 6c). It was the only fish in the tank besides the spawning pair that had a swollen papilla. Both members of the pair attacked it regularly in between bouts of egg-laying and fertilizing. Repeatedly, immediately after the female made an egg-laying pass over the spawning site, the small male dove towards the spawning site. Semen could not be seen in the water. The small male also exhibited satellite male behavior – it attacked other males that came near the spawning site. Around 3:00 P.M. the pair finished spawning and the papillae of all three fish regressed and the small fish ceased diving toward the spawning site. The behavior was observed again in September 2013. Videos documenting the behavior have been permanently publicly archived online at Case Western Reserve University's Digital Case electronic archive (URL pending).

Parental care

The total numbers of brooding females attended by males vs. brooding females unattended by males differed significantly between wild *H. minckleyi* and wild *H. cyanoguttatus* (Table 2). Reproductive *H. cyanoguttatus* females were always observed with a male mate, while reproductive *H. minckleyi* females were often observed without a male mate (Figure 7). Total numbers of females attended by males vs. females unattended by males differed significantly between species when controlling for reproductive stage (Mantel-Haenszel-Cochran test: $\chi^2_{MH} = 9.571_{(1)}$, $P = 0.002$), i.e., the difference in male presence between *H. minckleyi* and *H. cyanoguttatus* is not due to

differences at particular offspring ages. We also compared the distribution of unattended and attended female *H. minckleyi* in the early stages of reproduction with those at the late stage of reproduction and found no significant difference (Fisher's exact test: $P = 0.680$), i.e., the analysis cannot say that *H. minckleyi* males are present less often as their offspring get older (Table 2). These results indicate that although male *H. minckleyi* did not constantly associate with a particular brooding female, they maintained ongoing relationships with those females with which they had mated. Finally, the pattern observed in the artificial ponds in Austin, Texas was similar to the pattern observed in Cuatro Ciénegas, indicating that the tendency of males to be absent from the nest is an evolved trait and not a phenotypically plastic response to the particular environment of Cuatro Ciénegas.

The raw behavior data are given in Appendix A. The MANOVA that tested the effects of the independent variables on total aggression and distance to nest/offspring found three significant effects as shown by Wilks' Lambda (Sex: $\lambda_{2, 41} = 0.811$, $P = 0.014$; Offspring stage: $\lambda_{2, 41} = 0.530$, $P < 0.001$; Sex*Offspring stage: $\lambda_{2, 41} = 0.846$, $P = 0.032$), which justified additional analyses with univariate tests. ANOVAs and Poisson GLMs found attacks and distance to nest/offspring to differ among cells (Table 3). When males and females were considered together, there was no significant difference between species in number of attacks performed, but when males and females were considered separately, there was a significant difference (there was a significant interaction between sex and species). The results of the simple effects tests are shown in Fig. 8. After correction for multiple comparisons (Benjamini & Hochberg, 1995), the only simple

effect that remained significant was the difference in number of attacks performed between males and females of *H. minckleyi* when guarding old (free-swimming) offspring. However, we noted all differences of $p < 0.05$ in the graphs in attempt to explain the strongly significant effects revealed by the univariate AVOVAs and Poisson GLMs. In summary, male *H. minckleyi* (when present) performed fewer attacks than did male *H. cyanoguttatus* and female *H. minckleyi* (with a male present) when defending free-swimming fry. This difference between the sexes was not present in *H. cyanoguttatus* (Fig. 8a). In addition, in the presence of a male and in the early stages of reproduction, female *H. minckleyi* performed more attacks than female *H. cyanoguttatus* (Fig. 8a). There were no significant differences in frequency of aggressive displays in either species (Fig. 8b). Males of both species significantly reduced their distance to offspring when the offspring became free swimming (Fig. 8c).

Univariate ANOVAs and Poisson GLMs comparing brooding *H. minckleyi* females not attended by a male with brooding female *H. minckleyi* that were attended by a male revealed a significant interaction between reproductive stage and male presence in producing aggressive displays (Table 4). After correction for multiple comparisons (Benjamini & Hochberg, 1995), none of the simple effects remained significant. However, we noted all differences of $p < 0.05$ in the graphs in attempt to explain the strongly significant effects revealed by the univariate AVOVAs and Poisson GLMs. Female *H. minckleyi* in the early stages of reproduction performed more aggressive displays in the absence of a male than in the presence of a male, and more than females with free-swimming fry in the absence of a male (Fig. 9). In addition, female *H.*

minckleyi in the early stages of reproduction were closer to the nest when the male was absent than when the male was present (Mann-Whitney: $W = 25.0$, $N1 = 6$, $N2 = 6$, $P = 0.0306$, Fig. 9c). Finally, there was some inconsistency between the results of the ANOVA and the Poisson GLM. The Poisson GLM indicated strong significant effects of both male presence and of offspring state, and their interaction, on number of attacks performed, while the ANOVA indicated no significant effects. Simple effects tests also showed no significant differences among cells. Inspection of Fig. 9a reveals nearly equal means and a great variation in numbers of attacks performed, so it seems likely that the Poisson GLM was overly liberal in assigning significant differences to this variable and that the ANOVA result of no significant difference is probably more accurate.

Discussion

We have found that characteristics of body color, mating system, and parental care behavior in *H. minckleyi* differ remarkably from characteristics typically observed in other *Herichthys* species. The unusual trophic dimorphism in pharyngeal jaw morphology in *H. minckleyi* has received attention from ecologists and evolutionary biologists for decades. The details of the unusual aspects of reproductive biology of *H. minckleyi* reported here highlight even further its potential as a model species for studies of evolution, and point out the need for even closer study of this species to more fully understand its biology.

Our observations on breeding color were mostly consistent with Kornfield and Taylor (1983), although they did not report the sexually monochromatic patterns we saw

in some breeding individuals. Our phylogenetic analysis produced a topology consistent with previous studies (Hulsey et al., 2010; McMahan et al., 2010; de la Maza-Benignos et al., 2014; Fig. 1), and our ancestral state reconstruction indicated that strong sexual dichromatism evolved in the lineage of *H. minckleyi* after its separation from other species. This suggests that some aspect of the environment of Cuatro Ciénegas selected for the remarkable differences in body color between males and females.

Sexual dichromatism is often a result of sexual selection, which is typically stronger in polygynous mating systems than in monogamous mating systems (Cunningham & Birkhead, 1998; Seehausen, 1999; Badyaev & Hill, 2003). Sexually dimorphic traits may arise via two mechanisms of sexual selection: intra-sexual selection or intersexual selection, or reasons other than sexual selection (Slatkin, 1984; Shine, 1989). Under intra-sexual selection, male-male competition may result in the evolution of traits that communicate competitive ability, but that might not be preferred by females (Moore & Moore, 1999). Under inter-sexual selection, female preference may drive evolution of a trait that is exaggerated in males, or exhibited only in males. Our observations suggest that the all-black color in male *H. minckleyi* might be a result of intra-sexual selection in contests between rival males. An association between dark coloration and aggressive behavior was demonstrated previously by Neil (1984), who found in *T. meeki*, which does not have an all-black display but does have dark-barred displays, that fish engaged in agonistic activity were more likely to exhibit dark patterns than light patterns. Furthermore, a change to one of the dark barred patterns (which was very rapid, occurring in 1 sec.) was often sufficient to cause an intruder to leave the area

with no further agonistic activity. The all-black color in *H. minckleyi* is easily visible against the white substrate of Cuatro Ciénegas and could serve a similar purpose, but we cannot rule out that females may have preferences for all-black color. The all-black body color was rarely observed in captive males, possibly due to the lack of white substrate, or because individuals more easily establish dominance and eliminate rivalry under typical captive conditions, which lack sufficient environmental complexity to allow subordinate males to rise in social status (Hofmann et al., 1999; Oldfield, 2011). The snowy white color color pattern exhibited in breeding females (and some breeding males) seemed to correspond to the “striped” color pattern described by Barlow (1976) for *Amphilophus* species, in which the lateral spots are darkened in a row along the side of the fish, and may also be a result of intra-sexual selection. Compared to a monogamous mating system in *H. cyanoguttatus*, the mating system in *H. minckleyi* may elicit greater competition among females for male parental care. The white colored form of females (and some males) may match the common white substrate of Cuatro Ciénegas but contrasts with the typical all-black color pattern of large, territorial males, providing a clear signal of a female’s reproductive status.

In *H. minckleyi*, we found a presumably sexually selected trait (all-black body color) and an ecologically selected trait (molariform pharyngeal jaws) not to be correlated among individuals, indicating that they evolved independently. Artigas-Azas (1994) suggested that in the wild, black body color was exhibited by molariform individuals during feeding. Artigas-Azas (1996) also observed black body coloration during feeding in *Nosferatu labridens*, one of only two additional *Herichthys* or *Nosferatu* species that

674 also exhibits robust molariform pharyngeal teeth (de la Maza-Benignos & Lozano-
675 Vilano, 2013; de la Maza-Benignos et al., 2014; Oldfield, personal observations). That
676 suggests that the tendency to exhibit all-black body color may occur as a pleiotropic
677 effect of the gene(s) that codes molariform dentition. Interestingly, sexual selection has
678 been proposed to interact with ecological selection to promote speciation in sympatry.
679 One model states that after local adaptation through ecological selection occurs, mate
680 choice preferences then arise for ornaments that signal the adaptation and the resulting
681 assortative mating prevents gene flow (Turner & Burrows, 1995; Podos, 2001; van Doorn
682 et al., 2009). Such a model might explain rapid evolution in some cichlid groups
683 (Schliewen et al., 2001; Salzburger, 2009); Wagner et al. (2012) found that coincidence
684 between natural selection and sexual selection together predicted adaptive radiation in
685 African cichlids. The different pharyngeal morphs of *H. minckleyi* have previously been
686 reported to interbreed and to be genetically homogenous at some loci (Kornfield et al.,
687 1982), but a genetic component to pharyngeal jaw morphology has been demonstrated
688 (Trapani, 2003c), suggesting that if all-black body color has the potential to serve as a
689 signal of molariform dentition then mating preference for body color could drive the
690 future reproductive isolation of the two *H. minckleyi* pharyngeal morphs. Because both
691 pharyngeal jaw morphology and body color are dimorphic in *H. minckleyi*, we were able
692 to test for an association between the two traits at the species level. Our results did not
693 support an interaction between natural selection and sexual selection. Instead, it seems
694 likely that when *H. minckleyi* colonized Cuatro Ciénegas the same ecological release that
695 allowed trophic diversification simultaneously and independently allowed male

monopolization of females, sexual selection, mating system diversification, and evolution of visual signals. Perhaps the lack of association between natural selection and sexual selection is related to the fact that *H. minckleyi* has not yet radiated into multiple species in Cuatro Ciénegas.

While the current study has provided an improved understanding of body color patterns in *H. minckleyi*, clearly there remains much to be learned. In both males and females, which breeding color pattern was exhibited was not associated with presence of offspring: males and females of each color pattern were observed with offspring. Is color pattern independent of offspring but dependent on mate and competitor interactions? Also, it is not known if all individual males are capable of producing all color patterns observed, or if different males are genetically predisposed to exhibiting different breeding color patterns, perhaps in a situation similar to the dichromatism among males that exists in many African cichlids (Dijkstra, 2008). Additionally, while apparently rare, oligomelanic individuals have been observed before (Konings, 1994) and may be under positive selective pressure. Whereas oligomelanic coloration is well understood in at least one heroine species (Barlow, 1983), it is not clear what forces might select oligomelanic individuals of *H. minckleyi* in Cuatro Ciénegas. Obviously, body color is complex in *H. minckleyi* and will probably require controlled behavioral experiments to better understand.

In Cuatro Ciénegas, we confirmed reports by Kornfield et al. (1982) of large, all-black males guarding large territories that included ≥ 1 female and in Texas our observations of *H. cyanoguttatus* were similar to those reported by Buchanan (1971) and

Itzkowitz & Nyby (1982). We did not have sufficient data to perform an ancestral state reconstruction for mating system, but several observations suggest that polygynous mating behavior evolved in *H. minckleyi* after it colonized Cuatro Ciénegas: (1) many *Herichthys* and *Nosferatu* species have been reported to form monogamous pairs and no other species in either of these genera have been reported to be polygynous, (2) monogamy is nearly ubiquitous throughout the tribe Heroini (Goodwin et al., 1998), and (3) sexual dichromatism is often associated with polygyny (Seehausen, 1999; Badyaev & Hill, 2003) and our ancestral state reconstruction of body color found that strong sexual dichromatism evolved in the lineage of *H. minckleyi*. Our observations of polygynous mating in *H. minckleyi* in the artificial ponds in Austin, Texas, after being 17 years removed from Cuatro Ciénegas, indicate that polygynous mating in *H. minckleyi* is the result of a true evolutionary change in behavior and is not simply a result of phenotypically plastic behavioral adjustment to local conditions in Cuatro Ciénegas. An evolutionary transition from monogamy to polygyny is consistent with the pattern often found in fishes: a progression from no parental care to male-only care to monogamous biparental care to polygynous female-only care (Gross & Sargent, 1985). This pattern has previously been observed in phylogenetic analyses of several lineages of African and South American cichlids (Goodwin et al., 1998; Gonzalez-Voyer et al., 2008), although transitions from female-only care to biparental care have also occurred (Kidd et al., 2012).

Additionally, we provided photographic and video graphic evidence of alternative male mating behavior in *H. minckleyi*, providing the first report of sneaking and satellite

behavior in a heroine cichlid. In mating systems in which a small number of typically large, dominant males monopolize access to females, alternative mating tactics often evolve to allow other males to achieve successful fertilizations (Gross, 1996). These often take the form of a subordinate male sneaking into the territory of a large, dominant male and stealing fertilizations with a female that is attempting to spawn with the dominant male (Taborsky, 1994). Further research will be necessary to determine if the tendency to perform alternative male mating behavior is genetically selected or behaviorally plastic. Alternative mating tactics are well represented in fishes, and in cichlids in particular (Kato et al., 2005), but is a surprising discovery in a heroine cichlid considering their typically monogamous mating systems.

Our prediction (1) that, in *H. minckleyi*, males would accompany females and offspring less often than in *H. cyanoguttatus*, was supported. Males of *H. minckleyi* attended females and offspring less often than did males of *H. cyanoguttatus*. In *H. cyanoguttatus* every brooding female was attended by a male, both in the current study and in Itzkowitz & Nyby (1982). In *H. minckleyi* there was no difference in male presence between females in the early stages of reproduction and females with free-swimming fry. This suggests that males of *H. minckleyi* did not desert their mates and offspring, but maintained on-going relationships with those females with whom they had mated, consistent with a polygynous mating system. An alternative explanation is that some males do not desert females and that those males who desert females do so early in the reproductive cycle but not later. However, our observations in the artificial ponds suggest that this is not the case; of the eight large territorial males observed over a period

of several weeks none regularly maintained close proximity to a particular female (Oldfield et al., 2013).

Our prediction (2) that males of *H. minckleyi* would perform lower quantitative rates of parental care behavior in the form of brood defense and also maintain a greater distance from the brood site than would males of *H. cyanoguttatus* was also supported. Even by considering only those males that were present at a brood site, we found that males of *H. minckleyi* performed less indirect parental care in the form of offspring defense than did males of *H. cyanoguttatus*. In *H. cyanoguttatus* we did not find that males performed less aggression than females as Itzkowitz & Nyby (1982) found, but we did see such a difference in *H. minckleyi*. Differences in parental care between the sexes were expected to be greater in *H. minckleyi* due to the greater sexual conflict that would be expected in a polygynous mating system than in a monogamous mating system (Davies, 1989; Westneat & Sargent, 1996).

Prediction 3 was also supported. Females of *H. minckleyi* compensated for the low levels of defense contributed by the males by performing more attacks than females of *H. cyanoguttatus*, even when females of *H. minckleyi* that were unaccompanied by males were excluded from the analysis (Westneat & Sargent, 1996). Furthermore, despite the fact that males of *H. minckleyi* seemed to make little contribution to offspring defense, their female mates worked even harder when they were absent by performing an increased number of aggressive displays and remaining closer to the nest. This apparently plastic adjustment in behavior is consistent with Itzkowitz's (1984) laboratory observations that both males and females of *H. cyanoguttatus* reduced their distance to

the brood after their mate was experimentally removed. Similar observations have been made in other cichlids (Townshend & Wootton, 1985; Yanagisawa, 1985; van den Berghe & McKaye, 2001; Morley & Balshine, 2002; Lehtonen, 2011b; O’Connell et al., 2012). Interestingly, males of both species significantly reduced their distance to offspring when the offspring became free swimming, as was reported in captive lone male and lone female *H. cyanoguttatus* (Itzkowitz, 1985). This may be a spatial effect of tending offspring at different developmental stages: eggs or non-motile wrigglers occupy a compact area a few cm in diameter but free-swimming fry form a school several dm in diameter that often envelops a tending parent. Rates of aggression overall were similar to the rates reported by Itzkowitz & Nyby (1982) of 10 “chases” per 15 min. Although these comparisons in behavior involve only two species, the consistent pattern of significant differences we observed in several aspects of behavior as well as in physiology and gene transcription (Oldfield et al., 2013) suggest that they reflect evolutionary changes and not merely random differences between species (Garland & Adolph, 1994).

Large territories and multiple mates may have been selected in males of *H. minckleyi* as a result of relatively low levels of competition and/or offspring predation in Cuatro Ciénegas (Davies, 1989). In some habitats in Central America (and also in Lake Tanganyika, Africa) there may be so many cichlid species that defense by both a female and a male are required for some species to successfully obtain a suitable spawning site (McKaye, 1977; Morley & Balshine, 2002), resulting in selection for monogamous mating behavior. For example, male Midas cichlids, *Amphilophus citrinellus* (Günther, 1864), did not desert their mates even when presented with sex ratios biased toward

806 females (Rogers, 1987). For heroines in some other environments, there is no shortage of
807 spawning sites but both parents are nevertheless required to defend their offspring from
808 predation (Neil, 1984; Townshend & Wootton, 1985). Mate removal experiments, and
809 disturbances which cause the male to flee, typically result in some offspring being eaten
810 (Neil, 1984; Yanagisawa, 1985; Nagoshi, 1987; Keenleyside & Mackereth, 1992). A
811 similar high level of predation threat likely exists for *H. cyanoguttatus* in the San Marcos
812 River, where darters, *Etheostoma fonticola*, have been observed to quickly devour eggs
813 after the parents were chased away by an observer (Buchanan, 1971). However, under
814 reduced competition or predation, a male may seek additional mates if the fitness gained
815 exceeds the fitness lost by reducing care to existing offspring (Gross, 2005). These
816 factors as well as female-biased sex ratios are known to cause some typically
817 monogamous male cichlids to abandon their mates (Buchanan, 1971; Keenleyside, 1983;
818 Townshend & Wootton, 1985; Keenleyside et al., 1990; Wisenden, 1994). An alternative
819 to mate desertion is to acquire additional mates while maintaining a relationship with an
820 existing mate (Keenleyside, 1991). At the sites in Cuatro Ciénegas where *H. minckleyi*
821 occurs, it is the only native cichlid. Low levels of competition may have allowed *H.*
822 *minckleyi* to evolve trophic dimorphism in response to novel food sources (Swanson et
823 al., 2003; Cohen et al., 2005; Hulsey et al., 2006), and may also have been involved in the
824 evolution of polygynous males. Low levels of competition and predation, combined with
825 the stable environment of Cuatro Ciénegas (constant spring-derived water levels,
826 autochthonous food sources, consistently clear water that could allow males to visually
827 survey larger territories) may have selected dominant males that maintain large long-term

polygynous territories instead of small temporary monogamous territories. Additionally, high food availability might allow females to energetically perform a higher rate of brood defense, relieving males of the need to provide care. The only heroine cichlid other than *H. minckleyi* reported to typically be polygynous under natural conditions is *Parachromis dovii* (Günther, 1864) (van den Berghe & McKaye, 2001), in which large body size may enhance females' ability to defend offspring thereby liberating males from the need to do so (Lehtonen et al., 2011a).

During their initial invasion of Cuatro Ciénegas, in the absence of other cichlid species, a large number of ancestral male *H. minckleyi* might have been free to compete among each other for breeding territories. The resulting high level of intra-specific, intra-sexual competition may have caused the difference in mating success between highly competitive (and possibly sexually dichromatic) males and less competitive males to increase (i.e., sexual selection would increase). Because of the constant conditions in Cuatro Ciénegas, highly competitive males might form long-term territories, pre-empting space from males that are less competitive and thereby causing a reduction in the number of territorial, reproductively active males. If a male's territory were large enough, it could encompass the territories of multiple females, resulting in a polygynous mating system, evolution of alternative mating tactics in males, and an evolutionary reduction in male parental care rates.

In conclusion, our quantitative comparison of behavior, rooted in a phylogenetic context, accounts for feedback effects that might occur during long term evolutionary changes in mating system (Kokko & Jennions 2008, 2012; Alonzo, 2010) and allows

consideration of specific circumstances surrounding the evolution of body color, mating system, and parental care. Finally, it highlights the remarkable biology of the Cuatro Ciénegas cichlid and, because mating system is crucial to minimum viable population size (Nunney & Campbell, 1993), it also highlights important implications regarding its conservation (IUCN, 2013).

Acknowledgements We thank Laura Alberici da Barbiano, Chelsea Blake, Caitlin Gabor, Jorge L. Hurtado-Gonzales, Michael Jennions, Hanna Kokko, Diane Neudorf, and Amanda Winn for helpful comments on the manuscript. The Texas Natural History Collections at the University of Texas at Austin and the University of Michigan Museum of Zoology provided access to specimens. This work was supported by an Engineering Plus National Science Foundation -ADVANCE Opportunity grant (R.G.O.), by the Teresa Lozano Long Institute of Latin American Studies at the University of Texas at Austin from funds granted to the Institute by the Andrew W. Mellon Foundation (D.A.H.), by National Science Foundation grant DEB-0916695 (P.C.), and by National Science Foundation Grant IOS-0843712, an Alfred P. Sloan Foundation Fellowship, the Dwight W. and Blanche Faye Reeder Centennial Fellowship in Systematic and Evolutionary Biology, and an Institute for Cellular & Molecular Biology Fellowship (H.A.H.).

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1164 **Figure legends**

1165 **Fig. 1** Phylogeny and ancestral state reconstruction of body color in *Herichthys* and
1166 *Nosferatu* cichlid species and an outgroup cichlid species (*Thorichthys meeki*). Open
1167 circles represent sexual monochromatism or weak sexual dichromatism, which is
1168 ancestral, and a black filled circle represents strong sexual dichromatism, which is
1169 derived. A hashed circle indicates that information was not available for a particular
1170 species and that character state is uncertain. Percentages shown at select nodes (for the
1171 ancestor of *H. minckleyi* and its closest relatives, and for the ancestor of all species of
1172 *Herichthys* and *Nosferatu*) show the probability that the node has the plesiomorphic state
1173 (monochromatism).

1174

1175 **Fig 2** Non-breeding color patterns observed in *H. minckleyi*. Background color ranged
1176 from (a) light gray to tan, green, and (b) yellow. (c) Oligomelanic morphs caught in the
1177 wild and (d) acquired albinism in an aquarium specimen.

1178

1179 **Fig 3** Breeding color patterns observed in *Herichthys* species. (a) Breeding color
1180 typically observed in monogamous *Herichthys* species. Shown is a young *H.*
1181 *cyanoguttatus* female brooding offspring in an aquarium. At this time, vertical bars were
1182 accentuated and the darkened patch was not as solidly black as is sometimes observed.
1183 (b) A young breeding male *H. cyanoguttatus* in an aquarium. The anterior portion often
1184 does not turn as white in the male as it does in the female, instead retaining the normal
1185 gray background. (c) A captive female *H. minckleyi* exhibiting breeding colors typical of

other *Herichthys* species. This pattern was observed both in captivity and in nature. (d) A captive male *H. minckleyi* exhibiting a color pattern similar to that typically observed in breeding individuals of other *Herichthys* species, with the exception that the anterior half of the body retained the usual grayish-green background instead of lightening to contrast with the posterior portion. (e) Male and female breeding pair with free-swimming offspring and both exhibiting the white body color pattern, which was often exhibited in breeding females of *H. minckleyi*, both in Cuatro Ciénegas and in captivity. Each black vertical bar is reduced to a lateral spot (Barlow, 1976). This color pattern was also observed in males in Cuatro Ciénegas but not in captivity. (f) All-black body color in males exhibited during a competitive male-male interaction (lateral display). This color pattern was also sometimes observed in males attending females and in other non-reproductive individuals. (g) A large black male pictured immediately after competing with two other males for an insect prey. (h) The same male seconds later. After it got the prey it fled and turned green.

Fig 4 Test for association between pharyngeal jaw morphology and all-black breeding color. (a) At the phylogenetic level, all-black body color appears to be associated with molariform pharyngeal morphology. (b) If body color is truly linked with pharyngeal jaw morphology in *H. minckleyi*, then only individuals exhibiting molariform pharyngeal jaws will exhibit all-black body color, and sexual selection might facilitate speciation. (c) If the two dimorphisms evolved independently, then molariform individuals may or may not exhibit all-black body color. We found no statistical association between pharyngeal

1208 jaw morphology and black body color (see text).

1209

1210 **Fig 5** Territoriality in *Herichthys minckleyi* and *H. cyanoguttatus*. (a) A large black male
1211 in Cuatro Ciénegas attending a large nest (hole in the substrate) near a white
1212 (reproductively colored) female. Directly above in the photograph, another
1213 reproductively colored female is hovering over another nest, and on the right is a third
1214 reproductively colored female. (b) *Herichthys minckleyi* males in artificial ponds in Texas
1215 formed large territories that encompassed one or more breeding females and smaller
1216 males and females. (c) Wild *H. cyanoguttatus* males and females formed a pair bond and
1217 together defended a small, temporary territory from which they excluded all other fishes.
1218 b and c taken from Oldfield et al. (2013).

1219

1220 **Fig. 6** Alternative male mating behavior in *Herichthys minckleyi*. (a) The aquarium in
1221 which male sneaking behavior was observed. (b) A large, dominant male and female in
1222 the process of spawning on the side of the rock, with the sneaker hiding behind the log
1223 waiting to dash out after the female lays a batch of eggs. (c) Enlarged genital papilla in
1224 the sneaker male. Videos of the sneaking behavior have been permanently archived
1225 online (URL pending).

1226

1227 **Fig 7** Percentage of female *Herichthys cyanoguttatus* and *H. minckleyi* attended by a
1228 male at any time during a 15 min. observation period. *H. cyanoguttatus* data from Shoal
1229 Creek and Spring Lake, Texas. *H. minckleyi* data from artificial ponds in Texas and from

Cuatro Ciénegas, Mexico.

Fig. 8 Mean behavior of males (black columns) and females (white columns) of *H. cyanoguttatus* and *H. minckleyi* at different stages of reproduction. All individuals included were in the presence of a mate. Bars indicate standard deviation. Results of simple-effects tests (t-tests) for which $p < 0.05$ are shown by letters. (A) Indirect brood care / nest defense in the form of attacks toward other fishes. When guarding older, free-swimming offspring, males of *H. minckleyi* performed fewer attacks than their female mates and fewer than males of *H. cyanoguttatus*. When guarding eggs or young offspring, female of *H. minckleyi* performed more attacks than their *H. cyanoguttatus* counterparts. a: $t_5 = 2.87$, $P = 0.035$; b: $t_{11} = 2.29$, $P = 0.043$; c: $t_5 = 7.10$, $P = 0.001$. (B) Indirect brood care / nest defense in the form of displays toward other fishes. There were no significant differences between groups. (C) Mean distance to offspring. Males of both species were closer to their offspring when the offspring were free-swimming. a: $t_4 = 4.40$, $P = 0.012$., b: $t_5 = 3.12$, $P = 0.026$

Fig. 9 Mean behavior of females of *H. minckleyi* in the presence of a male (black columns) compared to females of *H. minckleyi* not attended by a male (white columns). Bars indicate standard deviation. (A) There were no significant differences between groups in indirect brood care in the form of attacks performed toward other fishes. (B) At early stages in the reproductive cycle unattended females defended the nest by displaying to other fishes more frequently than both paired females and unattended females with

1252 free-swimming offspring (a: $t_9 = 2.35$, $P = 0.043$; b: $t_9 = 2.51$, $P = 0.033$), (C) Distance to
1253 nest/offspring. In females of *H. minckleyi* that were brooding offspring that were not yet
1254 free-swimming, those without a male present remained closer to the offspring than did
1255 those that had a male present (a: $W = 25.0$, $P = 0.0306$).

1256 **Table 1** GenBank loci for sequences used in the phylogenetic analysis, and breeding color pattern and mating system reported
 1257 in literature for five species of *Herichthys*, four species of the closely related genus *Nosferatu*, and one heroine cichlid
 1258 outgroup species (*Thorichthys meeki*).

1259	Species	cytochrome b S7		Breeding color	Mating system
1260					
1261	<i>Thorichthys meeki</i>	HM193445	EF433025	Monochromatic ^{a, b}	Monogamous ^{a, b}
1262	<i>N. bartoni</i>	AY843355	GU736783	Monochromatic ^{c,d}	Monogamous ^c
1263	<i>N. labridens</i>	DQ990720	GU736782	Monochromatic ^{e,d}	Monogamous ^e
1264	<i>N. steindachneri</i>	AY324013	GU736787	? ^f	Monogamous ^e
1265	<i>N. pantostictus</i>	AY323989	GU736786	Monochromatic ^e	? ^g
1266	<i>H. cyanoguttatus</i>	AY323985	GU736781	Monochromatic ^h	Monogamous ^h
1267	<i>H. carpintis</i>	DQ990717	EF433019	Monochromatic ^{i,j}	? ^g
1268	<i>H. tamasopoensis</i>	DQ990716	GU736784	Monochromatic ^k	Monogamous ^k
1269	<i>H. deppii</i>	DQ494384	GU946320	Monochromatic ^l	? ^g
1270	<i>H. minckleyi</i>	AY323995	DQ836821	Dichromatic ^o	Polygynous ^{m,n}

1271 ^a Baerends and Baerends-van Roon, 1950; ^b Neil, 1984; ^c Artigas Azas, 1994; ^d Taylor & Miller, 1983; ^e Artigas Azas, 1992; ^f
 1272 Body color not available; ^g Mating system not available; ^h Buchanan, 1971; ⁱ Staeck & Linke, 1985; ^j Konings, 1989; ^k Artigas
 1273 Azas, 2006; ^l Lampert & Hanneman, 2002; ^m Kornfield et al., 1982; ⁿ Oldfield et al., 2013; ^o Kornfield & Taylor, 1983.

1274 **Table 2** Numbers of wild reproductively active *Herichthys* females that were observed either with or without a male mate
 1275 during a 15-min. observation period

		<i>H. cyanoguttatus</i>			<i>H. minckleyi</i>		
		n	# w/ male	# w/out male	n	# w/ male	# w/out male
		<hr/>					
1280	early stage	5	5	0	12	6	6
1281	late stage	10	10	0	14	6	8
1282	Total	15	15	0	26	12	14

1284 Female *H. cyanoguttatus* were observed with males significantly more often than were *H. minckleyi*, irrespective of
 1285 reproductive stage. Early stage: offspring not yet produced, eggs, or wrigglers. Late stage: free-swimming fry

Table 3 Results of univariate models comparing behavior in reproductive male and female monogamous *H. cyanoguttatus* and polygynous *H. minckleyi* at different stages of reproduction

ANOVA					Poisson GLM				
				Distance					
		Attacks		Displays		to offspring		Attacks*	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	χ^2	<i>P</i>
								χ^2	<i>P</i>
Omnibus test								156.85	<0.001
Species		2.95	0.093	1.56	0.218	1.72	0.197	24.02	<0.001
Sex		9.20	0.004	0.17	0.680	4.85	0.033	6.48	0.011
Stage		2.69	0.109	0.23	0.636	25.10	<0.001	48.14	<0.001
Species*Sex		4.96	0.031	0.35	0.555	1.28	0.264	4.86	0.028
Species*Stage		3.91	0.055	2.41	0.128	1.16	0.289	46.67	<0.001
Sex*Stage		0.35	0.560	0.61	0.441	5.18	0.028	4.33	0.037
Species*Sex*Stage		0.61	0.439	2.44	0.126	1.86	0.180	0.10	0.752

d.f. = 1, 42 for each ANOVA; d.f. = 7 for Poisson GLM omnibus; d.f. = 1 for each additional Poisson test. *P*-values < 0.05 are highlighted in bold font. All individuals analyzed were attended by a mate. The multivariate Wilks' lambda value was significant for several independent variables (see text). *analyzed with negative binomial distribution due to over dispersion

Table 4 Results of univariate tests comparing behavior in female *H. minckleyi* at different stages of reproduction and either unattended or in the presence of a male

ANOVA					Poisson GLM				
				Distance					
Attacks		Displays		to offspring		Attacks*		Displays	
<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
Omnibus test						62.94	<0.001	14.65	0.002
Male presence	0.08	0.777	0.64	0.434	not normal	13.45	<0.001	0.33	0.567
Stage	2.17	0.157	0.94	0.345	1.71 0.205	28.62	<0.001	0.40	0.527
Male presence*Stage	0.01	0.915	15.38	0.001	not normal	17.80	<0.001	12.46	<0.001

d.f. = 1, 20 for each ANOVA; d.f. = 3 for each omnibus test; d.f. = 1 for each additional Poisson test. Distance to offspring was not normally distributed for male presence. *P*-values < 0.05 in bold font. *analyzed with negative binomial distribution due to over dispersion

1321 **Appendix A** Raw values of numbers of aggressive attacks and displays performed, length of time observed (min.), and mean
1322 distance from nest / offspring, observed for wild *H. minckleyi* and *H. cyanoguttatus*.

1323	Species	Location	Offspring age	Sex	male present?	Attacks	Display	minutes	Distance
1324	<hr/>								
1325	<i>H. cyano.</i>	Spring Lake	not swimming	M		13	1	15	0.475
1326	<i>H. cyano.</i>		not swimming	F	yes	4	1	15	0.146
1327	<i>H. cyano.</i>	Spring Lake	swimming	M		6	0	15	0
1328	<i>H. cyano.</i>		swimming	F	yes	10	1	15	0
1329	<i>H. cyano.</i>	Spring Lake	swimming	M		8	4	15	0
1330	<i>H. cyano.</i>		swimming	F	yes	15	1	15	0.001
1331	<i>H. cyano.</i>	Spring Lake	swimming	M		0	0	15	0.051
1332	<i>H. cyano.</i>		swimming	F	yes	5	0	15	0.086
1333	<i>H. cyano.</i>	Spring Lake	swimming	M		0	1	15	0.036
1334	<i>H. cyano.</i>		swimming	F	yes	10	3	15	0.079
1335	<i>H. cyano.</i>	Shoal Creek	swimming	M		2	0	15	0.009
1336	<i>H. cyano.</i>		swimming	F	yes	3	1	15	0.002
1337	<i>H. cyano.</i>	Shoal Creek	not swimming	M		0	1	15	0.536
1338	<i>H. cyano.</i>		not swimming	F	yes	3	1	15	0.61
1339	<i>H. cyano.</i>	Shoal Creek	not swimming	M		2	24	15	0.134

1340	<i>H. cyano.</i>		not swimming	F	yes	1	14	15	0.129
1341	<i>H. cyano.</i>	Shoal Creek	swimming	M		1	5	15	0.003
1342	<i>H. cyano.</i>		swimming	F	yes	1	4	15	0.009
1343	<i>H. cyano.</i>	Shoal Creek	swimming	M		3	4	15	0.017
1344	<i>H. cyano.</i>		swimming	F	yes	7	0	15	0
1345	<i>H. cyano.</i>	Shoal Creek	not swimming	M		1	0	13	0.347
1346	<i>H. cyano.</i>		not swimming	F	yes	5	3	15	0.089
1347	<i>H. cyano.</i>	Shoal Creek	swimming	M		4	7	15	0.114
1348	<i>H. cyano.</i>		swimming	F	yes	2	3	15	0.024
1349	<i>H. cyano.</i>	Shoal Creek	swimming	M		1	8	15	0.064
1350	<i>H. cyano.</i>		swimming	F	yes	1	9	15	0.016
1351	<i>H. cyano.</i>	Shoal Creek	not swimming	M		6	3	15	0.214
1352	<i>H. cyano.</i>		not swimming	F	yes	3	9	15	0.1
1353	<i>H. cyano.</i>	Shoal Creek	swimming	M		2	3	15	0.034
1354	<i>H. cyano.</i>		swimming	F	yes	6	0	15	0.024
1355	<i>H. minck.</i>	Tortuguera	not swimming	F	no	7	4	15	0.01
1356	<i>H. minck.</i>	Tortuguera	not swimming	M		0	0	14.16	1.346
1357	<i>H. minck.</i>		not swimming	F	yes	5	3	15	0.059
1358	<i>H. minck.</i>	Tortuguera	swimming	F	no	2	4	15	0
1359	<i>H. minck.</i>	Tortuguera	not swimming	M		2	1	15	0.446
1360	<i>H. minck.</i>		not swimming	F	yes	5	0	15	0.158

1361	<i>H. minck.</i>	Tortuguera	not swimming	M		0	2	15	0.541
1362	<i>H. minck.</i>		not swimming	F	yes	10	1	15	0.082
1363	<i>H. minck.</i>	Tortuguera	swimming	F	no	0	0	15	0
1364	<i>H. minck.</i>	Mojarral Este	not swimming	M		0	2	15	2.312
1365	<i>H. minck.</i>		not swimming	F	yes	26	3	15	0.962
1366	<i>H. minck.</i>	Mojarral Este	swimming	F	no	32	1	15	0.046
1367	<i>H. minck.</i>	Mojarral Este	swimming	F	no	19	0	15	0.038
1368	<i>H. minck.</i>	Mojarral Este	not swimming	F	no	30	1	15	0.021
1369	<i>H. minck.</i>	Mojarral Este	swimming	F	no	13	1	15	0.039
1370	<i>H. minck.</i>	Rio Mesquites	not swimming	M		15	4	15	0.308
1371	<i>H. minck.</i>		not swimming	F	yes	15	0	15	0.052
1372	<i>H. minck.</i>	Rio Mesquites	swimming	F	no	3	1	13.84	0.011
1373	<i>H. minck.</i>	Rio Mesquites	not swimming	F	no	3	2	15	0.006
1374	<i>H. minck.</i>	Rio Mesquites	swimming	F	no	0	1	14.67	0.002
1375	<i>H. minck.</i>	Rio Mesquites	not swimming	F	no	0	3	15	0
1376	<i>H. minck.</i>	Rio Mesquites	not swimming	F	no	15	4	15	0.118
1377	<i>H. minck.</i>	Rio Mesquites	swimming	F	no	1	2	15	0.006
1378	<i>H. minck.</i>	Hundidos	swimming	M		0	0	15	0.09
1379	<i>H. minck.</i>		swimming	F	yes	6	3	15	0.03
1380	<i>H. minck.</i>		swimming	M		0	0	15	0.03
1381	<i>H. minck.</i>		swimming	F	yes	6	3	15	0.09

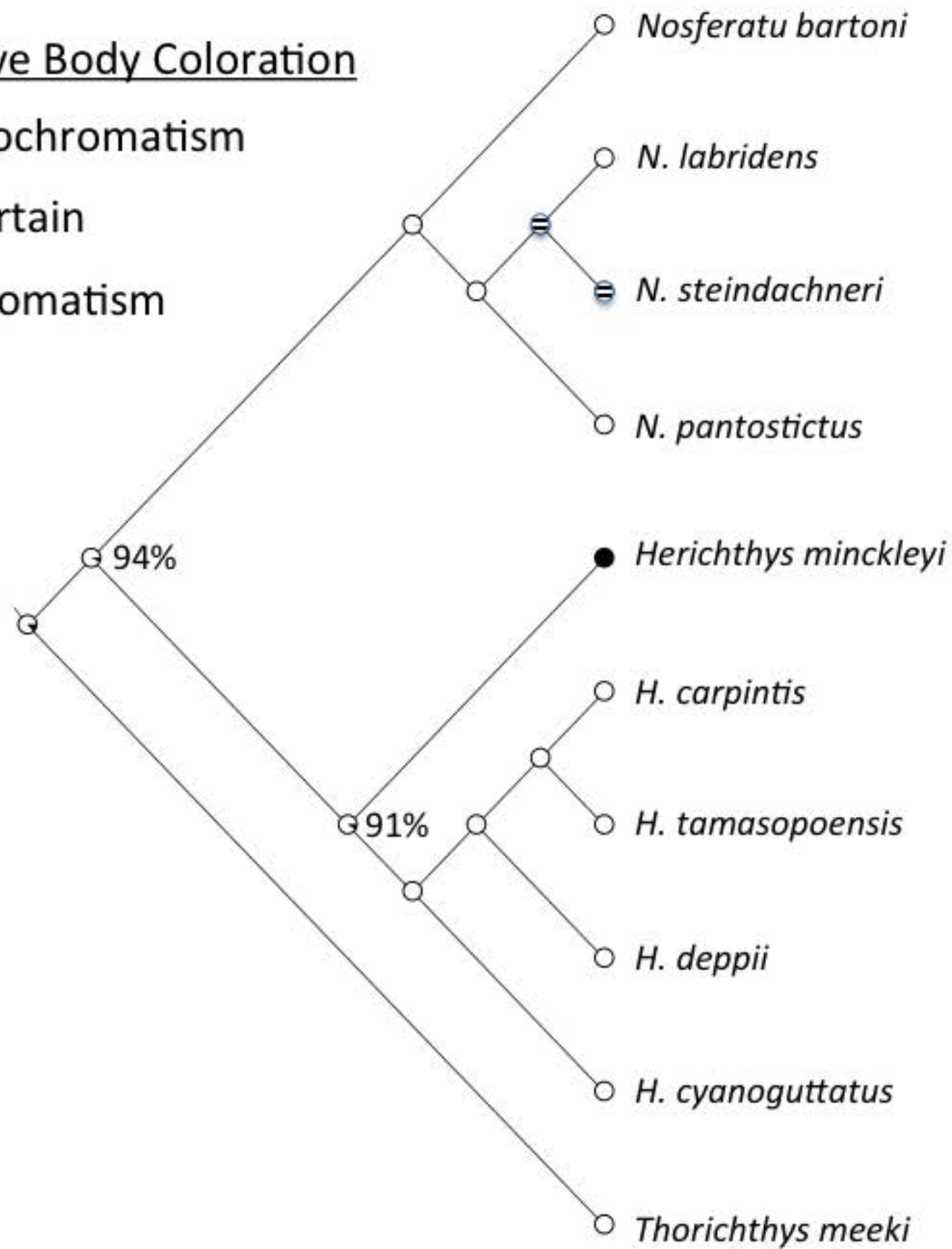
1382	<i>H. minck.</i>	Los Gatos	swimming	M		2	4	15	0.004
1383	<i>H. minck.</i>		swimming	F	yes	10	4	15	0.036
1384	<i>H. minck.</i>	Los Gatos	swimming	M		0	1	15	0.008
1385	<i>H. minck.</i>		swimming	F	yes	8	9	15	0.042
1386	<i>H. minck.</i>	Escobedo	not swimming	M		14	4	10	0.158
1387	<i>H. minck.</i>		not swimming	F	yes	27	0	10	0.055
1388	<i>H. minck.</i>	Escobedo	not swimming	F	no	41	6	15	0.014

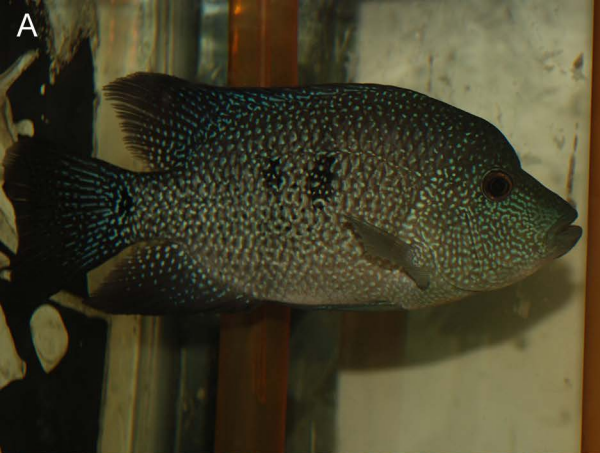
Reproductive Body Coloration

○ monochromatism

▨ uncertain

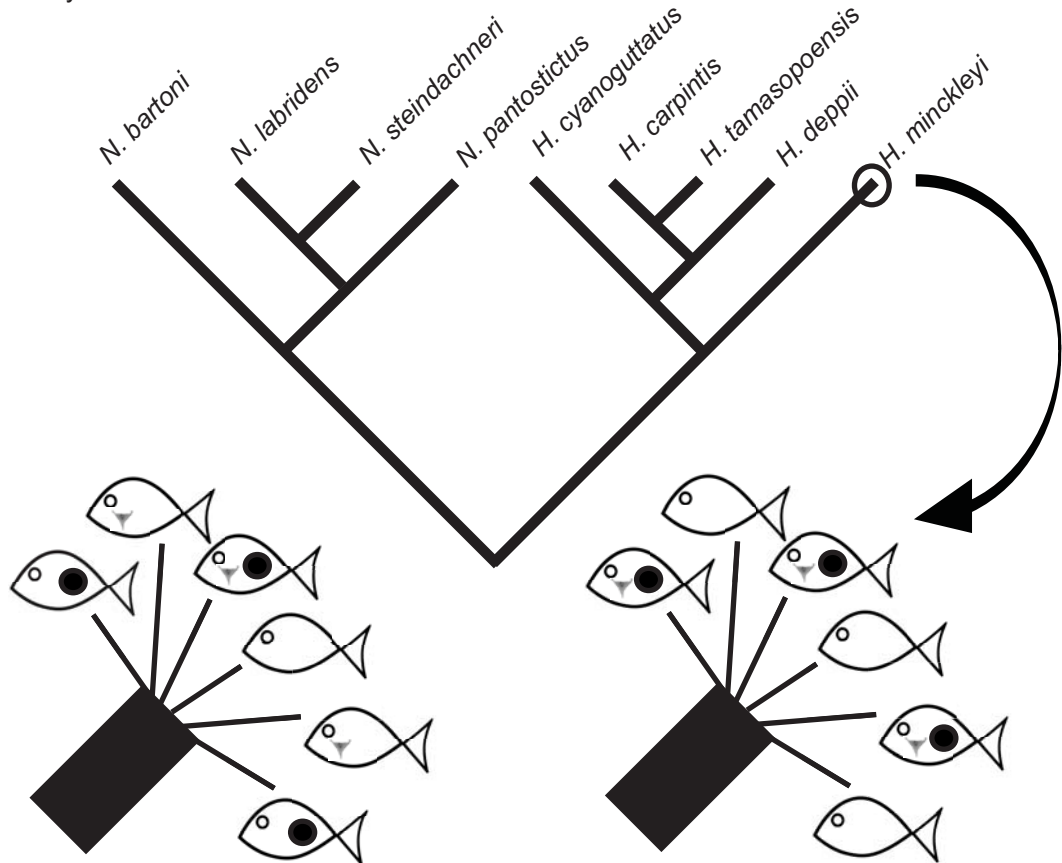
● dichromatism





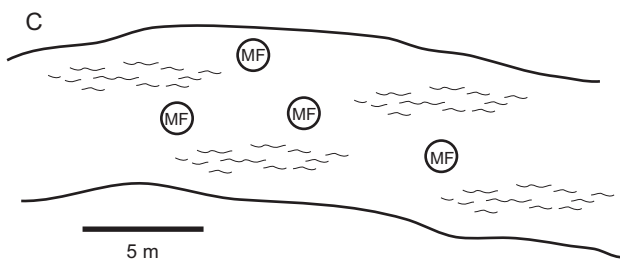
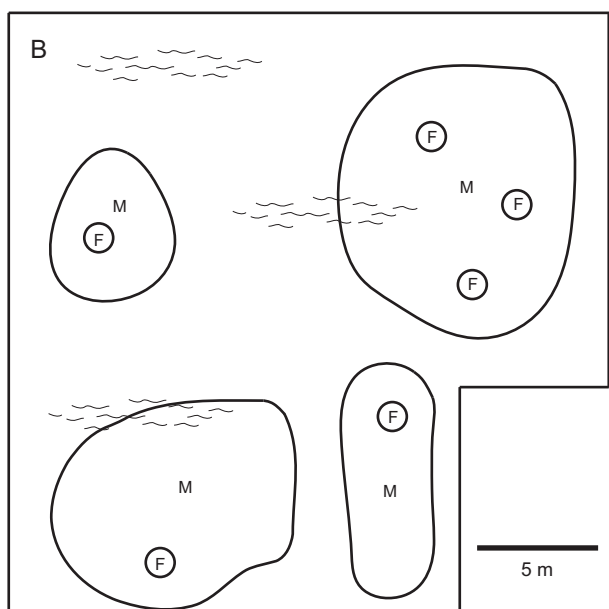


Molariform jaws	0	1	0	1	0	0	0	0	0/1
Black body color	0	1	0	0	0	0	0	0	0/1



No association between molariform jaws and all-black body color

Only those individuals that have molariform jaws also exhibit all-black color



A



B



C



