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# LIFE-HISTORY EVOLUTION IN LIVEBEARING FISHES (TELEOSTEI: POECILIIDAE)

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# ABSTRACT

Life histories lie at the heart of biology, because the tradeoffs each organism faces concerning the distribution of limited resources into either reproduction or maintenance and growth determine that organism's fitness. Generally speaking, an organism can choose to either invest in traits reducing age-specific mortality or to invest in traits increasing age-specific fecundity. Each decision in this regard leads to tradeoffs between current versus future reproduction, somatic maintenance or growth versus reproduction, or number, size and sex of offspring. Because of this, basic life history data provides the backbone for any organism-oriented research, effectively linking behavior, ecology, morphology, and physiology together. Not surprisingly, life-history evolution has therefore been an integral part of evolutionary research over the past decades.

One family of teleosts fishes, the livebearing fishes (Poeciliidae), have proven to be excellent models for studying life history adaptations, and probably the best-studied poeciliid is the guppy, *Poecilia reticulata*. Originally, the more than 200 species and 22-29 genera of poeciliids were endemic to the Americas, but due to human introductions they are now found worldwide. Within Poeciliidae, at least three characters evolved to give rise to the present diversity within the family: (1) internal fertilization using a transformed anal fin, referred to as the gonopodium, for sperm transfer, (2) livebearing, and (3) different degrees of maternal provisioning for the developing young. With the sole exception of *Tomeurus gracilis*, which is characterized by oviparous egg retention, internal fertilization and viviparity are found in all species of poeciliids.

Over the years, certain aspects of poeciliid life-history evolution have received particular attention. First and foremost, different predatory regimes have been demonstrated to drive rapid microevolutionary change in guppies, although evidence for macroevolutionary processes is as yet missing. Second, placental evolution and maternal provisioning strategies have been extensively studied, with the main focus of these studies on interspecific rather than intraspecific differences.

The research detailed in my dissertation investigates several specific aspects of life-history evolution in livebearing fishes (Poeciliidae) that have so far been largely neglected. The first three chapters broaden our understanding of the importance of life-history evolution in the colonization of and ecological speciation in extreme habitats. Taken together, they highlight the relevance of life-history evolution in maintaining and even driving ecological speciation processes. The fourth chapter emphasizes the significance of environmental affects and intraspecific population differences on maternal provisioning strategies in livebearing fishes. Thus, cautioning against the traditional approach of investigating few ecologically similar populations and ascribing the identified patterns as rigid strategies for that particular species across its natural distribution. Finally, my last chapter opens the door for research on life histories as regulatory mechanisms for stability in the unisexual/bisexual mating system of *Poecilia formosa*, the Amazon molly.

## *Extremophile poeciliids*

Although mechanisms that can lead to speciation are of fundamental importance to evolutionary biology, the actual process of speciation is still one of the least understood aspects of evolution. Recently, the idea that reproductive isolation and ultimately speciation can be the result of ecologically-based divergent selection (i.e., ecological speciation) has received a lot of attention. Two main components of ecological speciation are currently recognized: different sources of divergent selection, and different forms of reproductive isolation.

Basically, sources of divergent selection can stem from differences between environments, ecological interactions, and/or sexual selection. Under the first scenario, populations inhabit environments that, for example, differ in resource availability or habitat structure, and as populations adapt to their specific environment, the may begin to diverge from one another. Under the second scenario, divergent selection between populations arises due to ecological interactions, for example via competition for shared resources. Finally, under the third scenario, mate preferences differ between populations from ecologically different environments, thus leading to divergent sexual selection.

Different forms of reproductive isolation are usually distinguished based on whether they occur before (premating isolation) or after mating (postmating isolation). Premating isolation can for example evolve if populations are separated in time or space, or if populations are sexually isolated, i.e., populations simply differ in their mating signals and preferences. Postmating isolation on the other hand arises due to reduced hybrid fitness, which can originate from genetic incompatibilities, a mismatch between hybrid phenotypes and the environment, or a mismatch between hybrid phenotypes and the mating preferences of conspecifics.

Poeciliids inhabit a wide variety of different habitats, ranging from small creeks to large streams, freshwater lakes to coastal (brackish) lagoons, and even subterranean to toxic environments. In particular, poeciliid species of at least three genera (i.e., *Gambusia*, *Limia*, and *Poecilia*) are known to have independently colonized various toxic habitats in the Dominican Republic, México, and the United States. Some species, like *Gambusia eurystoma*, *Limia sulphurophila*, and *Poecilia sulphuraria* are even endemic to sulfidic waters, and via ecological speciation, are actually derived from poeciliids that can be found in sulfidic and nonsulfidic waters of adjacent habitats. In these systems, toxicity stems from naturally occurring hydrogen sulfide ($H_2S$), which is acutely toxic to most metazoans, because it competes with oxygen in the respiratory chain. Nonetheless, extremophile poeciliids thrive in these habitats and often occur in high local population densities.

A plateau in Tabasco, southern México, provides an even more extreme suit of habitats. Here, divergent selection stems from drastic differences between the physio-chemical characteristics of environments inhabited by populations of the Atlantic molly (*Poecilia mexicana*), and habitats can be grouped into four distinct classes: toxic/cave, nontoxic/cave, toxic/surface, and nontoxic/surface. All habitat types are interconnected (i.e., no physical barriers prevent movement between environments) and merely several hundred meters apart; nonetheless, locally adapted populations are characterized by profound genetic differentiation coupled with strong behavioral, morphological, and physiological divergence. Premating isolation seems to be largely driven by natural selection in the cave molly system, because translocation experiments revealed high mortalities in fish transferred to waters of a different adjacent habitat type. But even a weak form of sexual isolation has been demonstrated: females prefer males from their own populations over males from adjacent environments. However, the role of life histories in population divergence in this and similar systems has so far remained unstudied.

In my first chapter, I therefore began to address this issue by conducting a preliminary study that investigated differences in life-history evolution (i.e., fecundity) between *Poecilia mexicana* from the sulfidic Cueva del Azufre and from a benign surface habitat. Using a combination of data from field-collected and laboratory-reared animals, I was able to demonstrate that cave mollies not only exhibit reduced fecundity, but also that fecundity in cave mollies is less plastic than in their surface-dwelling counterparts. Although this clearly suggests a heritable component to fecundity divergence in cave mollies, the extent of total life history divergence between cave and surface mollies remained unexplored. Furthermore, these results led me to ask the following question: Was reduced fecundity a response to permanent darkness, to toxicity, or to the combination of both selective forces?

In my second chapter, I attempted to address these new questions. To do so, I conducted a full life-history analysis on females collected from four types of habitats, which provided me with a natural 2x2 design, where the same species inhabits environments characterized by all possible combinations of the two selective forces darkness and toxicity (i.e., dark/toxic vs. dark/nontoxic vs. light/toxic vs. light/nontoxic). The data demonstrate a habitat-specific divergence in *P. mexicana* life histories, and both darkness and toxicity seem to select for the same trait dynamic: low fecundity and large offspring size. This particular trait dynamic most likely arose as a strategy to avoid cannibalism in the extreme habitats, and to create a more efficient body-volume-to-body-surface-area ratio with regards to the amount of body surface exposed to toxicity. Thus, my third chapter adds further evidence to the notion of ecological speciation driving population differentiation between populations from these different habitat types. However, are any of these life-history patterns more generally applicable to life-history evolution and ecological speciation processes in poeciliids and other organisms, or is this type of divergence unique to the cave molly system?

In order for me to focus on this, I needed to take a broader approach. Hence, for my third chapter, I turned to another system of poeciliids in toxic waters: the Baños del Azufre system, in Tabasco, southern México. Here, two species (*Gambusia eurystoma* and *Poecilia sulphuraria*) have diverged, and eventually speciated, in sulfidic waters. Both of these sulfide-endemics derived from two more widespread poeciliids that inhabit surrounding nontoxic and toxic habitats (*Gambusia sexradiata* and *Poecilia mexicana*, respectively). In chapter 3, I report on a comparative analysis, in which I contrast life histories of all four species from different habitat types (*G. sexradiata*/nontoxic vs. *G. sexradiata*/incipient toxic vs. *G. eurystoma*/endemic toxic, and *P. mexicana*/nontoxic vs. *P. mexicana*/incipient toxic vs. *P. sulphuraria*/endemic toxic). Even though I also found evidence for genus-specific responses to toxicity, the most pronounced pattern was the same as in the cave molly: hydrogen sulfide induced low fecundity and large offspring size, and the higher the toxicity, the lower the fecundity and the larger the offspring. Overall, my first three chapters therefore provide me with evidence that poeciliid fishes colonizing sulfidic habitats exhibit convergent life history evolution, with the potential to eventually result in complete speciation (as is the case with *P. sulphuraria* and *G. eurystoma*). I therefore propose that after an initial period of trait divergence, life-history evolution may provide an additional mechanism for further divergence, because dispersers between habitats will suffer from reduced fitness due to the wrong life history strategy in the new environment. Hence, life histories can be an important mechanism for further divergence in the advanced stages of ecological speciation processes.

In my fourth chapter, I am turning to another important life-history aspect of livebearing animals; namely the question of maternal strategies for embryo provisioning. Basically, a mother has two choices: she can either store all the resources required for successful embryo development within the yolk prior to fertilization (lecithotrophy), or she can supplement yolk-stored nutrients with additional provisions via direct transfer after fertilization (matrotrophy). Even though recent theoretical models and empirical studies have stressed that maternal provisioning strategies are resource-dependent, mainstream life history research still attempts to classify an organism as being either exclusively lecithotrophic or matrotrophic. I therefore compared maternal provisioning strategies between two populations of *Poecilia mexicana* that inhabit vastly different environments: a toxic, resource-poor limestone cave and a benign, resource-rich surface habitat. Furthermore, I directly compared two different techniques that are widely used to quantify maternal provisioning: the indirect matrotrophy index analysis and the direct radio-tracer assay of maternal provisioning. According to the matrotrophy index analysis, both populations of *P. mexicana* are purely lecithotrophic, while according to the radio-tracer assay, both populations provide similar levels of postfertilization nutrient transfer (i.e., matrotrophy). Together with results from chapter 2, this suggests that *P. mexicana* is at least capable of incipient matrotrophy and that, to avoid misclassification, both techniques of quantifying nutrient transfer should ideally be employed together. Finally, I propose that current theories on the evolution of matrotrophy in poeciliids need to be revised, since this chapter and other recent research suggest that most livebearing fishes are probably characterized by dual provisioning rather than distinct strategies of either lecithotrophy or matrotrophy.

### *Unisexual poeciliids*

Unisexual vertebrates have long been used as model systems to study the maintenance of recombination and all species described to date originated as hybrids. Among unisexual vertebrates, fishes are of central interest because of their peculiar reproductive mechanisms. Several unisexual fishes reproduce via sperm-dependent parthenogenesis ('gynogenesis'), where sperm is required to initiate embryogenesis, but inheritance is strictly maternal.

In such mating systems involving a bisexual and a unisexual species we find two competing types of females, which rely on the same resource, sperm, but have fundamentally different population dynamics. According to theoretical models, the unisexuals should quickly outcompete the bisexuals, thus driving them to extinction. However, this would inevitably lead to their own subsequent demise. Hence, the stability of bisexual/unisexual mating systems and the factors underlying the maintenance of this stability are currently of great interest in evolutionary ecology.

The Amazon molly, *Poecilia formosa*, is a gynogenetic, all-female poeciliid. *Poecilia formosa* resulted from a single natural hybridization of the two sexual species *Poecilia latipinna* and *Poecilia mexicana*, and uses sperm from males of the two parental species for gynogenetic reproduction. Ecological differences and behavioral regulation have been proposed as two possible mechanisms explaining the stability in complexes of gynogens and sexuals, because at certain stages in their life history the gynogen could suffer a significant reduction of fitness. Since *P. latipinna* and *P. mexicana* males are not related to the resulting offspring from matings with Amazon mollies, they should be under selection to avoid them. Accordingly, both *P. latipinna* and *P. mexicana* males have been shown to (a) discriminate between heterospecific and conspecific females, and (b) to prefer to mate with conspecific females under most circumstances. However, these mating preferences are only of importance if they translate into reduced mating success of gynogens relative to conspecific females.

For my fifth and final chapter, I therefore investigated the mate choice:life history interface by documenting the presence of sperm in five natural populations. I extracted sperm from female fish of five syntopic populations of *P. formosa* and *P. latipinna* in Texas by flushing out the genital tract. A higher proportion of bisexual females had sperm than unisexuals. Also, among those females that had sperm, bisexuals had more sperm than unisexuals. Even though the results gained from this analysis cannot be used to infer the amount of sperm transferred per copulation or the number of heterospecific matings, they nonetheless show that Amazon mollies receive less sperm in the wild than Sailfin molly females. This represents the first study to investigate the ultimate effects of male mate choice as a stabilizing factor in natural populations, and my results suggest that *P. formosa* may in fact be sperm limited; however, future studies on life-history differences will have to determine whether this actually results in a fitness reduction.

# CHAPTER 1

# OFFSPRING NUMBER IN A LIVEBEARING FISH (*POECILIA MEXICANA*, POECILIIDAE): REDUCED FECUNDITY AND REDUCED PLASTICITY IN A POPULATION OF CAVE MOLLIES

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## ABSTRACT

Life history traits within species often vary among different habitats. We measured female fecundity in mollies (*Poecilia mexicana*, Poeciliidae) from a $H_2S$-rich cave and from a neighbouring surface habitat as well as in laboratory-reared individuals of both populations raised in either light or continuous darkness. Compared to conspecifics from surface habitats, cave-dwelling *P. mexicana* had reduced fecundity (adjusted for size) in the field. In the laboratory, the fecundity of surface mollies was higher in light than in darkness, whereas fecundity in the cave mollies was almost unaffected by the ambient light conditions. Our results suggest a heritable component to the reduction in fecundity in female cave mollies. Moreover, the reduced plasticity in fecundity of cave mollies in response to light conditions might be an example of genetic assimilation or channelling of a life history trait in a population invading a new environment.

## INTRODUCTION

Life history strategies vary widely across species, but also between different populations of the same species (Stearns 1992; Roff 2002). Within species variation in life history traits, which may be genetically fixed or phenotypically plastic, can be influenced by several environmental factors, such as resource availability, predation and stressful environmental conditions (Sibly and Calow 1989; Stearns 1992; Roff 2002). Cave-living organisms provide a unique opportunity to investigate life history evolution, because they have colonized a radically different habitat and in some cases the surface-dwelling ancestor is available for comparison. Life histories of cave animals are generally characterized by shifts towards longer life and generation times, older age at first reproduction, and increased brood care behaviour, as well as a decreased fecundity combined with an increase in individual offspring size (Collembola: Christiansen 1965; *Gammarus minus* (Amphipoda): Culver et al. 1995; Pisces: *Amblyopsis rosae* (Amblyopsidae): Winemiller 1992; *Astyanax mexicanus* (Characidae): Hüppop and Wilkens 1991; *Trichomycterus chaberti* (Trichomycteridae): Pouilly and Miranda 2003; for a review see Parzefall 2000). These traits were mostly interpreted as adaptations to food scarcity (Culver 1982; Hüppop 2000), because caves are usually nutrient-poor due to a lack of photoautotrophic primary production and reliance on nutrient-influx from epigean habitats (Poulson and Lavoie 2000). Caves, however, also differ from surface habitats in other factors known to influence life history traits, including relaxed predation (Romero and Green 2005; Tobler et al. 2006, 2007a) or the presence of suboptimal gas concentrations and toxicants (Howarth 1993; Tobler 2006); but importance of different selective forces in the evolution of life history strategies of cave dwellers is as yet poorly understood.

We studied life history traits in a small livebearing fish, the cave molly, a divergent population of the Atlantic molly (*Poecilia mexicana*, Poeciliidae) from the Cueva del Azufre in Tabasco, Mexico (Gordon and Rosen 1962; Parzefall 2001). The water in this particular cave contains high concentrations of toxic hydrogen sulphide ($H_2S$, up to 320 $\mu$M; Tobler et al. 2006). Migration into the cave from the outside is absent, and a source-sink relationship between surface and cave populations can be ruled out (Plath et al. 2007a). We compared the fecundity (size-corrected number of oocytes) of cave molly females with that of conspecifics from an adjacent non-sulphidic surface habitat using specimens collected in the field. Based on previous comparisons of epigean and hypogean organisms, we expected a reduction of fecundity (Christiansen 1965; Hüppop and Wilkens 1991; Winemiller 1992; Culver et al. 1995; Parzefall 2000; Pouilly and Miranda 2003). In a laboratory experiment, we further examined whether differences in fecundity between surface and cave mollies are due to plasticity in response to environmental conditions (presence or absence of light) rather than fixed traits. Our null-hypothesis was that the fecundity of both (cave and surface-dwelling molly females) would be fixed and that fecundity would not vary among the two treatment groups.

## METHODS

### *Study sites*

All fish used in this study originated from the Cueva del Azufre and adjacent waters near Tapijulapa (Tabasco, Mexico). The waters studied eventually drain into the Río Oxolotan and are located within a radius of about 5 kilometres. Within the cave, mollies were sampled from three chambers, V, X and XIII (Gordon and Rosen 1962), but data were pooled for analysis. For comparison, surface mollies were collected from a creek of similar size and structure lacking hydrogen sulphide (Arroyo Cristal). Details for the study sites can be found in Tobler et al. (2006).

### *Field study*

Females of both the surface and the cave populations were collected in August 2004 and January 2006 with seines and dip nets (Table 1 for sample sizes). Dip nets were used in the cave where low ceilings and rocky habitats preclude efficient seining. Fish were sacrificed with an overdose of MS 222 (Tricaine Methanesulfonate), and the standard length (SL) of each fish was measured. Then, the body cavity was opened and the number of ripe oocytes or developing embryos, if present, was counted.

Statistical analysis.-The frequency of gravid females was compared between habitats and years using a binary logistic regression where “gravid (yes/ no)” was the dependent variable and “population” and “year” were the independent variables. To test for differences in the fecundity of females between habitats and years, non-gravid females were excluded from the analysis, and data were square-root-transformed in order to achieve a normal distribution. Fecundity data was analyzed using a General Linear Model (GLM) with “number of oocytes (embryos)” as the dependent variable, “standard length” (SL) as a covariate, and “population” and “year” as independent variables. The interaction “population by year” was not significant ($F_1$=0.002, $P$=0.964), hence it was removed from the final model. Because of limitations in the field we did not attempt to stage or weigh the embryos. This will be reserved for future research. All statistical analyses were performed with SPSS 11 (SPSS Inc 2002).

### *Laboratory study*

Randomly out-bred laboratory populations of mollies, originally collected in the hindmost cave chamber XIII and the Río Oxolotan, were kept at the Biozentrum Grindel in Hamburg, Germany. These populations were established originally in the 1970s and repeatedly “refreshed” with wild-caught animals. Fish were reared and maintained in 100-200 l aquaria at 25-30°C. All populations were reared separately.

Fish were fed *ad libitum* twice daily with commercially available flake food, *Artemia* nauplii, water fleas, and *Tubifex* worms.

Ever since these stocks were established, fish were subjected to two treatments. Three to five stocktanks of each population were kept either in continuous darkness or under a normal day-night light cycle (16:8 h using artificial fluorescent light). Hence, each population was kept both in their natural light regime as well as in an artificial light regime. Besides the differences in the illumination, maintenance conditions were identical in the two experimental treatments. Like in the field study, females were sacrificed with an overdose of MS 222 for the assessment of fecundity. The SL of each fish was measured, the body cavity was opened, and the number of ripe oocytes or developing embryos was counted if present.

Statistical analysis.-The frequency of gravid females was compared between populations and light treatments using a binary logistic regression where "gravid (yes/ no)" was the dependent variable, and "population" and "light condition" (light vs. darkness) were independent variables. For the analysis of female fecundity, we employed a similar analysis as described above, but the independent variables of the GLM were "population" and "light condition" (light vs. darkness).

It is important to note that there were size differences for both populations between the field and the laboratory treatments (Table 1). One possible explanation for this is that the growth patterns between natural populations and our laboratory-reared populations were different (e.g., due to differences in type and amount of food). However, this size difference does not have any influence on our interpretation, because body size was included as a covariate in the analyses, and the relationship between female size and offspring number in both populations is expected to be linear for the observed size range (as found in the field data: Table 1).

## RESULTS

### *Field study*

Generally, a higher proportion of *P. mexicana* females from the cave were gravid as compared to fish from the surface habitat (Tables 1a, 2a). The frequency of gravid cave molly females was lower in January 2006 compared to August 2004. In surface dwelling females, it was higher in 2006 compared to 2004.

Cave molly females had significantly lower fecundity compared to conspecifics from surface habitats (Tables 1a, Table 3a). The fecundity of both females from the cave and the surface habitat increased with female standard length.

### *Laboratory study*

The laboratory experiment revealed significant differences in the frequency of gravid females among populations (Tables 1b, 2b). The interaction "population by light condition" was significant: In surface mollies a higher proportion of females was gravid in light than in darkness. In the cave molly, however, the difference in proportion gravid between light conditions was less pronounced (albeit not significantly), and was even slightly higher in darkness.

As in the field, fecundity increased with female standard length (Table 3b). In the GLM, cave mollies showed a trend of having a lower fecundity than surface mollies (Table 3b). There was a significant interaction between population and light condition. The fecundity of surface mollies was higher in light than in darkness, whereas fecundity in the cave mollies did not respond strongly to the light conditions (Table 1).

## DISCUSSION

In the field, cave mollies showed lower fecundity compared to conspecifics from surface habitats. In our experiment, however, where only the light conditions differed among treatments, fecundity differences among cave and surface populations were less pronounced. Whereas fecundity of surface mollies was reduced in continuous darkness as compared to normal light conditions, cave molly fecundity did not vary across the two experimental treatments. Our design provided very benign conditions because we provided *ad libitum* amounts of high quality food, and toxic $H_2S$, which is highly prevalent under natural conditions (Tobler et al. 2006), was absent. Such conditions should allow cave mollies to express the maximum of their reaction norm. Hence, differences between cave and surface forms observed in the field are not caused by entirely plastic responses to light conditions.

While surface-dwelling *P. mexicana* show phenotypic plasticity in fecundity, size-corrected fecundity of cave mollies did not vary in response to the light treatment suggesting that the fecundity reaction norm became much smaller in this population. Phenotypic plasticity in fecundity may be advantageous in variable surface habitats (Via 1993; de Jong 1995), but plasticity was lost in the more stable cave habitat. Thus, phenotypic plasticity in fecundity might have been genetically assimilated or channelled in response to the persistent expose of new environmental conditions (see Pigliucci 2005).

Incidentally, the reduction in fecundity is likely caused by selection on offspring size, which would constrain the maximum number of offspring a cave molly female could produce. Three different selective forces could be affecting fecundity directly and/ or indirectly: (1) Caves are often considered predator-free environments. Different predatory regimes have been shown to affect life history traits in the related guppy (Reznick and Endler 1982; Reznick et al. 1990). Indeed, the predatory regimes differ between hypogean and epigean habitats in that predatory fish and piscivorous birds are lacking in the cave (Tobler et al. 2006, 2007a), but the Cueva del Azufre is clearly not predator free. Giant water bugs (*Belostoma* sp.) are abundant in the cave system and are known to prey on mollies (Plath et al. 2003; Tobler et al. 2007b; Tobler et al. 2008). Therefore, lack of predation may not be responsible for the observed life history differences. (2) The Cueva del Azufre contains high amounts of toxic $H_2S$ (Tobler et al. 2006). Costly adaptations to excrete or neutralize toxins have been shown to have a profound influence on life history traits possibly by draining resources that could have been invested into reproduction (Sibly and Calow 1989). Indeed, short-term survival of cave mollies in sulphidic habitats critically depends on energy availability (Plath et al. 2007b), but physiological adaptations of cave mollies to $H_2S$ remain to be studied. (3) Resources may be limited in the cave. In fact, food limitation has been documented in many cave systems, and cave animals often evolve morphological and/or behavioural traits to cope with food scarcity (Hüppop 2000). Resource limitation has been proven experimentally to influence fecundity in guppies

(Reznick and Yang 1993; Reznick et al. 2001). The Cueva del Azufre was hypothesized to be rich in food resources because of bacterial chemoautotrophic primary production and the input of bat guano (Langecker et al. 1996), but a recent study has demonstrated reduced body condition in cave mollies compared to surface-dwelling fish from non-sulphidic waters (Tobler 2008). This may indicate that the detoxification of hydrogen sulphide requires a substantial amount of energy and/ or resource scarcity.

Current research will attempt to uncover differences in other life history traits in surface and cave mollies, the trade-offs between them and the extent to which they are plastic. The magnitude of fecundity differences among surface and cave mollies clearly highlights the value of this system in the understanding of life history evolution.

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**Table 1.** Descriptive statistics from the field study and the laboratory experiment. The size-corrected fecundity data refer to the estimated marginal means of the GLM and are in bold.

| | N | % gravid | fecundity (mean±SD) | fecundity (range) | SL of gravid females | **size-corrected fecundity (mean±SD)** |
|---|---|---|---|---|---|---|
| *a) Field data* | | | | | | |
| *Surface* | | | | | | |
| 2004 (August) | 59 | 20.3 | 21.3±10.3 | 4-45 | 47.7±5.3 | **3.9±0.7** |
| 2006 (January) | 29 | 27.6 | 24.5±11.0 | 11-47 | 52.5±10.9 | **3.9±0.8** |
| *Cave* | | | | | | |
| 2004 (August) | 48 | 75.0 | 3.0±1.4 | 1-6 | 35.6±3.7 | **2.0±0.7** |
| 2006 (January) | 23 | 47.8 | 4.2±3.2 | 1-11 | 38.7±6.2 | **2.0±0.6** |
| *b) Laboratory data* | | | | | | |
| *Surface* | | | | | | |
| Light | 29 | 89.7 | 8.5±5.6 | 2-23 | 35.6±5.3 | **2.4±0.5** |
| Darkness | 32 | 65.6 | 5.1±3.5 | 1-14 | 32.3±2.9 | **2.1±0.5** |
| *Cave* | | | | | | |
| Light | 67 | 71.6 | 4.3±2.6 | 1-13 | 32.6±4.1 | **2.0±0.5** |
| Darkness | 29 | 82.8 | 2.8±1.6 | 1-7 | 27.4±3.7 | **2.1±0.5** |

**Table 2.** Logistic regression on the frequency of gravid females. Significant *P*-values are given in bold.

| | -2 log likelihood | B | SE | Wald | df | *P* |
|---|---|---|---|---|---|---|
| *a) Field data* | 179.585 | | | | | |
| Population | | 1591.396 | 751.842 | 4.480 | 1 | **0.034** |
| Year | | 0.993 | 0.590 | 2.829 | 1 | 0.093 |
| Population*Year | | -0.793 | 0.375 | 4.470 | 1 | **0.034** |
| | | | | | | |
| *b) Laboratory data* | 167.041 | | | | | |
| Population | | -1.233 | 0.667 | 3.413 | 1 | 0.065 |
| Light condition | | -3.668 | 1.535 | 5.708 | 1 | **0.017** |
| Population*Light condition | | 2.155 | 0.909 | 5.624 | 1 | **0.018** |

**Table 3.** General linear model (GLM) on female fecundity (square root-transformed) in the field study and the laboratory experiment. SL=standard length. Significant *P*-values in bold.

| Factor | *df* | Mean Squares | *F* | *P* |
|---|---|---|---|---|
| *a) Field data* | | | | |
| SL | 1 | 11.401 | 31.370 | **<0.001** |
| Population | 1 | 22.505 | 61.923 | **<0.001** |
| Year | 1 | 0.000 | 0.000 | 0.983 |
| *b) Laboratory data* | | | | |
| SL | 1 | 25.681 | 109.338 | **<0.001** |
| Population | 1 | 0.903 | 3.847 | 0.052 |
| Light condition | 1 | 0.035 | 0.149 | 0.700 |
| Population*Light condition | 1 | 1.404 | 5.978 | **0.016** |

# CHAPTER 2

# TOXIC HYDROGEN SULFIDE AND DARK CAVES: LIFE–HISTORY ADAPTATIONS TO EXTREME ENVIRONMENTS IN A LIVEBEARING FISH (*POECILIA MEXICANA*, POECILIIDAE)

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# ABSTRACT

Life–history traits are very sensitive to extreme environmental conditions, because resources that need to be invested in somatic maintenance cannot be invested in reproduction. Here we examined female life–history traits in the Mexican livebearing fish *Poecilia mexicana* from a variety of benign surface habitats, a creek with naturally occurring toxic hydrogen sulfide ($H_2S$), and a sulfidic cave as well as a non–sulfidic cave. Previous studies revealed pronounced genetic and morphological divergence over very small geographic scales in this system despite the absence of physical barriers, suggesting that local adaptation to different combinations of the two selection factors toxicity ($H_2S$) and darkness is accompanied by very low rates of gene–flow. Our main results from the present study show that toxicity and darkness both select for (or impose constraints on) the same female trait dynamics: reduced fecundity and increased offspring size. Since reduced fecundity in the sulfur cave population was previously shown to be heritable, we discuss how divergent life–history evolution may promote further ecological divergence: for example, reduced fecundity and increased offspring autonomy are clearly beneficial in extreme environments, but are outcompeted in benign habitats.

# INTRODUCTION

## *Life–history traits in extreme environments*

Organisms that live in extreme habitats (e.g., due to toxins/toxicants) usually evolve costly adaptations to cope with the physiochemical stressors imposed by the environment (Townsend et al. 2003). When first encountered, these stressors lower an individual's fitness, and, thus, can have a profound influence on reproductive life–history strategies (Sibly and Calow 1989; Stearns 1992; Roff 2002). Since protecting oneself from a stressful environment by excreting or neutralizing toxins (for a review see Sibly and Calow 1989), or via behavioral adaptations (e.g., Plath et al. 2007*b*; Tobler et al. 2009*b*) is energetically costly, these processes decrease the resources and energy that can be invested elsewhere, e.g. into reproduction. Several studies have demonstrated phenotypic responses to toxins that exemplify the costs of dealing with environmental stress (guppies, *Poecilia reticulata*, Uviovo and Beatty 1979; *Onychiurus armatus*, Collembola, Bengtsson et al. 1985; pond snails *Lymnaea stagnalis*, Gérard et al. 2005).

Beside toxins and toxicants, perpetual darkness like in cave ecosystems can represent an extreme condition for typical surface–dwelling organisms (Tobler et al. 2006). Cave animals are widely used model organisms to study the evolutionary effects of permanent darkness (e.g., Culver 1982; Culver et al. 1995; Jeffery 2005), and their life–history traits are characterized by switches towards longer life and generation times, older age at first reproduction, increased brood care behavior, as well as decreased fecundity combined with an increase in individual offspring size (reviews in Parzefall 2000; Culver 2005). These traits are often interpreted as adaptations to continuous food scarcity (Culver 1982, 2005; Parker and Begon 1986; Hüppop 2000), because caves are usually nutrient–poor due to a lack of photoautotrophic primary production and reliance on nutrient–influx from epigean habitats (Poulson and Lavoie 2000).

### *Adaptive trait divergence in extremophile* Poecilia mexicana

On a plateau in Tabasco (southern Mexico), two populations of the wide–spread Atlantic molly (*Poecilia mexicana*) have colonized subterranean watercourses, the Cueva del Azufre (Gordon and Rosen 1962) and the Cueva Luna Azufre (Pisarowicz 2005; Tobler et al. 2008*c*). These two limestone caves are unique, because they are the only known caves inhabited by poeciliids. Moreover the watercourses of the cave plateau are characterized by the presence or absence of another physiochemical stressors: naturally occurring hydrogen sulfide ($H_2S$), and all combinations of surface/cave and non-sulfidic/sulfidic habitat types are found (Gordon and Rosen 1962; Tobler et al. 2008*c*). Hydrogen sulfide is acutely toxic to metazoans (Grieshaber and Völkel 1998) and leads to extreme hypoxia in the water (Tobler et al. 2006, 2009*b*). *Poecilia mexicana* from toxic habitats (both cave and surface) perform Aquatic Surface Respiration (ASR) to exploit the more oxygenated (and thus less sulfidic) top–most layer of the water column (Plath et al. 2007*b*; Tobler et al. 2009*a*). Hence, mollies from the cave plateau have to cope with the adverse effects of two strong selective forces: darkness and toxicity (Tobler et al. 2006, 2008*a*; Plath et al. 2007*a*).

Previous studies have demonstrated evolutionary responses of *P. mexicana* to these two environmental factors in morphological, physiological and behavioral traits (Zeiske 1968; Parzefall 1970; Tobler et al. 2008*a*). Common–garden rearing shows that divergence in the expression of several morphological traits clearly has a heritable basis, because they are also expressed when fish are reared in light for several generations (Parzefall 1970, 2001; Tobler et al. 2008*a*; Plath and Tobler 2010). Not surprisingly, ecological diversification even over extremely small spatial scales (sometimes only few hundred meters) is reflected by pronounced genetic differentiation (determined on the basis of nuclear microsatellite markers and mitochondrial DNA sequence divergence: Plath et al. 2007*a*; Tobler et al. 2008*a*). Gene flow between adjoining, but ecologically divergent habitat types on the cave plateau is indeed very low despite the absence of physical barriers (Plath et al. 2007*a*; Tobler et al. 2008*a*, 2009*b*).

Low gene flow suggests local adaptation to ecological gradients along two axes (1) non–sulfidic/sulfidic and (2) light/darkness, and strong selection against dispersing, non–adapted individuals (Nosil et al. 2005; Tobler et al. 2008*a*, 2009*b*). For example, laboratory–reared cave mollies from the Cueva del Azufre have a higher $H_2S$ resistance than fish from non–sulfidic surface streams (Peters et al. 1973; reanalyzed in Plath and Tobler 2010). Hence, this system provides a good opportunity to study the underlying mechanisms at work during ecological speciation (Rundle and Nosil 2005; Plath et al. 2007*a*; Tobler et al. 2008*a*; Tobler and Plath *in press*).

### *Life–history evolution in extremophile* P. mexicana

Since mechanisms that ensure survival in the face of stress can be demanding of resources, this can select for changes in life histories and cause local adaptation, ultimately leading to profound effects on fitness if dispersing individuals find themselves in a habitat type to which they are not locally adapted. However, except for a preliminary study on fecundity differences between the toxic cave and one of the surrounding non–toxic surface habitats (Riesch et al. 2009*b*), life histories have not been studied so far in this system. Here we examined female reproductive life–history traits in *P. mexicana* from four different habitat types that provided us with a natural 2 x 2 factorial design of two environmental stressors (darkness and toxic $H_2S$), where the same fish species inhabits all possible factor combinations. We asked two related questions: (1) What are the general and site-specific differences between the reproductive life–histories of *P. mexicana* from the four different habitat types? (2) Can we determine which life–history traits are selected for/constrained by toxicity and which by darkness?

## **METHODS**

### *Study system*

The Atlantic molly, *Poecilia mexicana*, is a widespread freshwater fish, living along the Atlantic coastal drainages of Mexico, and it exhibits wide habitat tolerances: It can be found in first through third order streams, creeks, brooks, lakes, springs, coastal lagoons, as well as in fresh to brackish waters (Miller 2005). There is also some evidence for the existence of two subspecies: *P. mexicana limantouri* inhabiting

Northern Mexico and *P. mexicana mexicana* occurring south of central Mexico (e.g., Menzel and Darnell 1973; Turner et al. 1983; but see Brett and Turner 1983), however support for both is still ambiguous and these designations are rarely used in the literature. Two populations of *Poecilia mexicana* (the so–called cave mollies) have colonized limestone caves near Tapijulapa in Tabasco, southern Mexico: the Cueva del Azufre and the Cueva Luna Azufre (Gordon and Rosen 1962; Tobler et al. 2008*c*). Inside the Cueva del Azufre, several springs discharge water that is rich in hydrogen sulfide ($H_2S$), so cave mollies in the Cueva del Azufre not only live in permanent darkness (Parzefall 2001), but also have to cope with the adverse effects of a naturally occurring toxicant (Tobler et al. 2006) and thus face two strong selective forces (Plath et al. 2007*a*; Tobler et al. 2008*a*). Recently, mollies have also been described from a second, non–sulfidic cave, the Cueva Luna Azufre (Pisarowicz 2005; Tobler et al. 2008*c*). This cave is located only a few hundred meters from the Cueva del Azufre, but a small valley separates the two caves and there is no gene flow between them (Tobler et al. 2008*a*). Even though the water inside the Luna Azufre is non–sulfidic, the inhabiting mollies face permanent darkness as a potentially strong selective force (Tobler et al. 2008*a*).

Both caves drain into the El Azufre, which is a sulfidic surface creek that contains high concentrations of hydrogen sulfide. Several sulfidic and non–sulfidic springs discharge into the El Azufre (also upstream from the Cueva del Azufre), which meanders through the valley separating both caves and eventually drains into the Río Oxolotán (Tobler et al. 2008*a*). The Río Oxolotán river system consists of numerous non–sulfidic surface habitats (including the Río Amatan and Arroyo Bonita sites sampled for this study) on and around the cave plateau. Fish communities in the Río Oxolotán and its tributaries are complex, but *P. mexicana* appears to be the only fish capable of coping with the conditions of all habitats, including the three described extreme habitats (Tobler et al. 2006, 2008*c*). Both caves, the El Azufre and the Río Oxolotán river system are part of the Río Grijalva/Usumacinta drainage (Miller 2005).

### *Study populations*

Individual *P. mexicana* were collected in June 2007 and January 2008 in and around the caves as well as in September 2007 in northeastern Mexico, and field preserved in 10% formaldehyde. Cave mollies were collected in the cave chambers V and X of the Cueva del Azufre (Gordon and Rosen 1962) and in the accessible parts of the Cueva Luna Azufre (Tobler et al. 2008*c*). Surface fish were collected from the sulfidic surface stream (El Azufre), and the non–sulfidic Río Amatan and Arroyo Bonita. The Arroyo Bonita is a tributary of the Río Oxolotán that is of similar size and structure as the El Azufre. The Río Amatan, on the other hand, is a typical river. It joins the Río Oxolotán, into which also El Azufre drains, approximately 2 kilometers downstream from the sample site (for a map, GPS coordinates and a distance matrix of these sites refer to Tobler et al. 2008*a*). Henceforth, the Arroyo Bonita and Río Amatan sites will be referred to as the southern Mexican (SM) sites.

We included several sites from northeastern Mexico (NM) as independent examples of *P. mexicana* populations from non–sulfidic, normoxic surface systems to examine if life–histories of the extremophile populations from the cave plateau deviate from the usual within–species variation. *Poecilia mexicana* were collected from two different drainages: (1) the Río Soto La Marina drainage [$NM_1$, two sites: Río Purificación at Barretal (24° 4'42.85"N, 99° 7'21.76"W) and Río Purificación at Nuevo Padilla (24° 2'35.59"N, 98°54'15.98"W)], and (2) the Río Tamesí/Río Pánuco drainage [$NM_2$, two sites: Río La Bomba (22°32'27.32"N, 98° 9'40.01"W) and an irrigation ditch west of Ebano along MX70 (22° 9'0.74"N, 98°30'12.36"W)] (Figure 1). Our northernmost sampling points in the subtropics (NM1) were located approximately 1,500 km north of the tropical cave plateau in Tabasco (Figure 1).

In summary, our sampling scheme included four different habitat types and provided us with a natural 2 x 2 design, where the same fish species inhabits all possible combinations of the two environmental factors “light” and “toxicity”: (1) $NM_1$, $NM_2$, and SM (all light/non–toxic), (2) El Azufre (light/toxic), (3) Luna Azufre (dark/non–toxic), and (4) Cueva del Azufre (dark/toxic).

### *Life–history analyses*

Following the protocol of Reznick and Endler (1982), all preserved fish were weighed and measured for standard length. The reproductive tissue and, if present, all developing offspring were removed. Offspring were counted and their stage of development determined (Reznick 1981; Reznick and Endler 1982; Haynes 1995; Reznick et al. 2002). Somatic tissues, reproductive tissues and embryos were then dried for 10 days at 40°C and weighed again. To assess female and embryo condition, somatic tissues and embryos were rinsed six times for at least six hours in petroleum ether to extract soluble non–structural fats (Heulett et al. 1995; Marsh-Matthews et al. 2005), and were then re–dried and re–weighed. Furthermore, we calculated reproductive allocation (RA) by dividing offspring weight by the sum of offspring weight plus somatic dry weight (Reznick and Endler 1982).

### *Maternal provisioning*

To evaluate the mode of maternal provisioning, the “matrotrophy index” (MI) was calculated (utilizing the slopes and regression coefficients from the regression analysis described below). The MI equals the estimated dry weight of the embryo at birth divided by the estimated dry weight of the oocyte at fertilization (e.g., Reznick et al. 2007). If the eggs are fully provisioned by yolk prior to fertilization (lecithotrophy), then we would expect the embryos to lose 35–40% of their dry weight during development (MI between 0.60–0.65; Scrimshaw 1945; Wourms et al. 1988). On the other hand, in the case of continuous maternal provisioning even after fertilization (matrotrophy), one would expect the embryos to lose less weight (MI between 0.65–1.00), or even to gain weight during development (MI $\geq$ 1.00; e.g., Reznick et al. 2002).

### *Statistical analyses*

All statistical analyses were conducted using SPSS 16.0.2 for Mac (SPSS Inc. 2008).

General linear models (GLMs). –We tested for differences in female condition between habitats and drainages by means of a factorial multivariate GLM (MANCOVA) with "female lean weight [g]” and “female fat content [%]” as dependent variables, “female standard length [mm]” as a covariate, “light condition (light vs. darkness)", and "toxicity (non–toxic vs. toxic)" as independent variables, and "drainage (SM vs. $NM_1$ vs. $NM_2$)" as a random factor.

Furthermore, we tested for differences between habitats and drainages in reproductive life–history traits using a similar model with "fecundity (number of offspring)", "embryo lean weight [mg]", "embryo fat content [%]", and "reproductive allocation [%]" as dependent variables, "female standard length (SL)", "female fat content [%]", and "embryo stage" as covariates, "light condition" and "toxicity" as independent variables, and "drainage" as a random factor.

All dependent variables and covariates were either log–transformed or arcsine–transformed (in case of percentages) to accommodate for potential non–linear relationships between the variables.

Discriminant function analyses (DFAs).–To provide an intuitive metric with respect to the magnitude of life–history divergence, we conducted several discriminant function analyses (DFA). We used a jackknife ('leave–one–out') sampling scheme as a cross–validation technique (i.e., each case is classified by the functions derived from all cases other than that case). For each DFA, *a priori* probabilities were calculated based on group sizes, and an overall classification success was calculated.

The first DFA tested for the discrimination power of life–history parameters along the two environmental factors "toxicity" and "light condition" as well as along geographic distance. The grouping variables were "Cueva del Azufre (dark/toxic)", "Luna Azufre (dark/non–toxic)", "El Azufre (light/toxic)", "SM (light/non–toxic)", "$NM_1$ (light/non–toxic)", and "$NM_2$ (light/non–toxic)". To accommodate the potential effects of "standard length (SL)" and "embryo stage" on life history traits, we used residuals from a preparatory GLM as dependent variables (note that the preparatory GLM used here is different from the analytical GLM described in the online supplement). In this multivariate GLM, “SL” and “embryo stage” were included as covariates. The dependent variables for the GLM and their residuals for the DFA were “female lean weight [g]”, “female fat content [%]”, “fecundity [number of offspring]”, “reproductive allocation [%]”, “embryo lean weight [mg]”, and “embryo fat content [%]”.

In a second set of DFAs, we tested for classification success based on habitat type within southern Mexican populations alone. The same dependent variables (as residuals) were used as in the previous analysis; however, this time the grouping variables were “dark/toxic (Cueva del Azufre)”, “dark/non–toxic (Luna Azufre)”, “light/toxic (El Azufre)”, and “light/non–toxic (SM)”. Again, the preparatory multivariate GLM included the covariates “SL” and “embryo stage”.

For all preparatory multivariate GLMs, all dependent variables and covariates were either log–transformed or arcsine–transformed (in the case of percentages) to control for potential non–linear relationships between the variables.

Principal components analysis (PCA).–We conducted PCA to test for classification based on life–history characters without defining groups prior to analysis. The dependent variables (“female lean weight [g]”, “female fat content [%]”, “fecundity [number of offspring]”, “reproductive allocation [%]”, “embryo lean weight [mg]”, and “embryo fat content [%]”) were again the residuals from a preparatory multivariate GLM with the factor “drainage” and the covariates “SL” and “embryo stage”.

### *Maternal provisioning (Matrotrophy index)*

Maternal provisioning was evaluated by analyzing the relationship between "embryonic dry weight" and "stage of development" by means of linear regression analysis (e.g., Reznick et al. 2002). ANCOVA was used to test for slope heterogeneity.

## RESULTS

Condition.–As predicted, the covariate standard length (SL) had a significant effect on female lean weight with larger females being heavier (Table A1). Light condition had an effect on female lean weight and toxicity had an effect on female fat content (Table A1). If the northern Mexican habitats were neglected, female condition (measured as fat content) was lowest in the toxic habitats (El Azufre: 2.74 %; Cueva del Azufre: 6.21 %) but was distinctly higher in the non–toxic environments (Luna Azufre: 8.52 %; SM: 9.93 %; Table 1 – main manuscript). Female fat content showed more variation between populations from southern Mexico (range of means: 2.74–9.93 %) than between benign surface populations across Mexico (3.47–9.93 %); however, fish from extreme environments did not exhibit lower fat contents than found among those from benign surface habitats (Table 1). Female lean weight showed the opposite pattern and was generally lower in extreme habitats (southern Mexico: 0.13–0.31 g; benign surface populations: 0.31–0.71 g; Table 1).

The interaction of "light condition by toxicity" had a significant effect on both variables (Table A1). Within surface habitats the decrease of female fat content from non–toxic to toxic was from 9.93 % (SM) to 2.74 % (El Azufre), while the decrease from non–toxic to toxic within caves was only from 8.52 % (Luna Azufre) to 6.21 % (Cueva del Azufre; Table 1 – main manuscript). In contrast, female lean weight decreased in surface habitats from non–toxic to toxic habitats (0.31 g in SM to 0.17 g in El Azufre) while it increased in the caves from non–toxic to toxic populations (from 0.13 g in the Luna Azufre to 0.20 g in the Cueva del Azufre; Table 1 – main manuscript).

Furthermore, we found an interaction effect of ”light condition by SL” on female lean weight (Table A1). Comparing linear regressions between female lean weight and SL (residuals corrected for drainage and toxicity) revealed that female lean weight increased more strongly with increasing SL in caves ($R$ = +0.971) than in surface habitats ($R$ = +0.722).

Reproduction.–All reproductive traits showed higher variability within southern Mexican populations (SM and extreme habitats) than between populations from benign surface habitats across Mexico (e.g., range of mean fecundity: 5.79–20.14 vs. 9.96–20.14, embryo fat content: 14.12–20.75 % vs. 16.37–20.75 %; Table 1). The lowest fecundity was found in females from the toxic Cueva del Azufre (5.79 offspring per female, corrected for size), and the highest in females from SM (20.14 offspring per female; Table 1). Whereas offspring number (fecundity) decreased in the dark habitats, offspring size (embryo lean weight) increased greatly (see estimated embryo dry weight at birth in Table 1). Embryo fat content was lowest in the caves and highest in the surface habitats (Table 1). On the other hand, offspring from sulfidic habitats were heavier than offspring from non–sulfidic habitats (see estimated embryo dry weight at birth in Table 1), with offspring from the sulfidic Cueva del Azufre being twice as large as the largest offspring from benign surface habitats.

In the GLM, standard length had a significant influence on fecundity and embryo lean weight (e.g., with large females having a higher fecundity and bigger embryos than smaller females; Table A1 and Table 1 – main manuscript).

Light condition had a significant effect on all variables: Females from cave habitats had significantly lower fecundity, heavier embryos, and embryos with lower body condition than females from surface habitats. Toxicity affected only fecundity, embryo lean weight, and reproductive allocation: Females in toxic habitats had reduced fecundity, heavier babies, and slightly increased reproductive allocation (Table A1 and Table 1 – main manuscript). Although we corrected for the effects of female condition and female size, we detected significant effects of drainage on reproductive allocation (RA). This difference in RA was largely driven by high values in the two toxic southern Mexican sites: Cueva del Azufre (13.73 %) and El Azufre (12.29 %; Table 1 – main manuscript).

The significant influence of light condition on RA was less straightforward: while females from the non–toxic cave had the lowest RA (Luna Azufre: 9.93 %), females from the toxic Cueva del Azufre had the highest (13.73 %; Table 1 – main manuscript). This was reflected by a significant interaction effect of ‘light condition by toxicity’ on reproductive allotment (Table A1; see below).

Additionally, we detected a significant effect of “drainage by embryo stage” on RA. When analyzing linear regressions between embryo stage and RA (residuals corrected for SL, female fat content, light condition, and toxicity) we found RA to strongly increase with embryo stage in $NM_1$ ($R$ = +0.513) and $NM_2$ ($R$ = +0.408), while it increased only slightly with embryo stage in SM ($R$ = +0.051).

Finally, we found a significant interaction effect of "light condition by toxicity" on fecundity, embryo fat content, and RA (Table A1). This indicates that the observed effects of light condition and toxicity are not independent of each other. While fecundity generally decreased from non-toxic to toxic habitats, this decrease was relatively small in surface habitats (20.14 in SM to 14.67 in El Azufre), but strong in caves (12.89 in Luna Azufre to 5.79 in Cueva del Azufre; Table 1 – main manuscript). Furthermore, embryo fat content decreased from 20.75 % in the non–toxic SM to 18.22 % in the El Azufre, the opposite pattern was observed within the cave habitats, where embryo fat content increased from 14.12 % in the non–toxic Luna Azufre to 15.75 % in the toxic Cueva del Azufre (Table 1 – main manuscript). In the case of reproductive allocation, the values increased from non–toxic to toxic habitats; however in surface habitats the increase was considerably weaker (from 11.18 to 12.29 %) than within the caves (from 9.93 to 13.73 %; Table 1 – main manuscript).

Discriminant function analyses (DFAs).–The first DFA (based on extreme habitats and non–extreme surface drainages) classified 75.5 % of the females into the correct group (Figure 2A). The variables with the most discriminatory ability were "embryo lean weight" (*Wilks' lambda* = 0.441, $F$ = 50.266, $P$ < 0.001) and "fecundity" (*Wilks' lambda* = 0.511, $F$ = 37.874, $P$ < 0.001). Figure 2A shows that discriminant function 1 mainly separates cave from surface habitats, while discriminant function 2 at a tendency seems to correspond to the separation based on geographic distance. Interestingly, the El Azufre population is clearly separated from the closest non–sulfidic surface habitat (SM), but rather grouped together with the two northern Mexican drainages $NM_1$ and $NM_2$. This disrupts the otherwise clear separation of northern from southern Mexican sites along discriminant function 2.

The second DFA was based on habitat type within southern Mexican populations alone and classified 82.8 % of the females into the correct group (Figure 2B). The variables with the most discriminatory power were again "embryo lean weight" (*Wilks' lambda* = 0.416, $F$ = 71.589, $P < 0.001$) and "fecundity" (*Wilks' lambda* = 0.439, $F$ = 65.070, $P < 0.001$). Figure 2B shows that discriminant function 1 mainly separates cave from surface habitats, while discriminant function 2 corresponds to the separation of toxic vs. non–toxic habitats.

As a *post–hoc* analysis, we re–ran the second DFA twice to test for the independent effects of first toxicity only (two groups: toxic vs. non–toxic habitats) and second light condition only (two groups: dark vs. light habitats). Based on toxicity alone, the classification success increased to 86.0 % ("embryo lean weight": *Wilks' lambda* = 0.661, $F$ = 79.322, $P < 0.001$; "fecundity": *Wilks' lambda* = 0.757, $F$ = 49.729, $P < 0.001$), but classification based on light condition alone was to 93.6 % successful ("fecundity": *Wilks' lambda* = 0.557, $F$ = 123.101, $P < 0.001$; "embryo lean weight": *Wilks' lambda* = 0.619, $F$ = 95.526, $P < 0.001$).

Principal components analysis (PCA).–The strong differentiation between cave and surface habitats was clearly reflected in the PCA (principal component 1), whereas differentiation based on toxicity was rather weak (Figure 3). The first two components of the PCA accounted for 62% of the variance. Like in the DFAs and GLMs (see online supplementary material), the two life–history variables that accounted for most of the variation along principal component 1 were “embryo lean weight” and “fecundity” (Figure 3).

### *Maternal provisioning*

In all six populations of *P. mexicana*, embryo dry weight decreased during embryo development (Figure 4); however, only in the Cueva del Azufre ($R^2 = 0.413$, $p < 0.001$), SM ($R^2 = 0.149$, $p = 0.002$), and $NM_2$ ($R^2 = 0.150$, $p = 0.034$) collections was this decrease statistically significant. The estimated MI was highest in the Luna Azufre (0.85) and lowest in the Cueva del Azufre (0.56) and SM (0.57) whereas all other drainages/extreme habitats ranged between 0.61 and 0.67 (Table 1; Figure 4). Differences in the decrease of embryo dry weight were reflected in a significant interaction of ‘drainage/extreme habitat by embryo stage’ (ANCOVA on log-transformed data: $F_{5,180} = 3.854$, $p = 0.002$).

## DISCUSSION

### *Life–history differentiation in extremophile* P. mexicana

In southern Mexico, *P. mexicana* has colonized four different habitat types. This provided us with a “natural 2 x 2 design”, where the same fish species inhabits watercourses with all possible combinations of the two abiotic environmental factors “toxicity” ($H_2S$) and “light/darkness”. Unsurprisingly, we found considerable variation in life–history traits comparing benign surface habitats across Mexico, which are up to 1,500 km apart from one another and in separate climate zones (subtropical in northern Mexico vs. tropical in southern Mexico). However, the pronounced variation seen in extremophile *P. mexicana* on a very small geographic scale on the cave plateau (several 100 meters) clearly follows a different selective trajectory. Hence, we argue that the observed pattern of strongly divergent life histories in southern Mexican populations is not one of 'isolation–by–distance' (Slatkin 1993), but rather caused by 'isolation–by–adaptation' (Nosil et al. 2008, 2009). Along with previous studies focusing on divergence in other traits (e.g., morphological or behavioral divergence), our current results support the hypothesis that "toxicity" and "darkness" are examples of two strong selective forces that shape adaptations for immediate survival (Plath et al. 2007*b*; Tobler et al. 2008*a*, 2009*b*) and successful reproduction (this study). Consequently, our discussion will focus primarily on differences between extremophile and non–extremophile *P. mexicana* from southern Mexico; analysis of general life–history variation in *P. mexicana* along biogeographic regions will be the focus of another study, including more populations across Mexico (R. Riesch, *unpublished data*).

*Poecilia mexicana* from the four different habitat types [(1) light/non–toxic, (2) light/toxic, (3) dark/non–toxic, and (4) dark/toxic] exhibited vast differences in various life–history traits. Even though a number of female life–history traits showed variation between habitat types, fecundity and offspring size were the traits that diverged most strongly (see also online supplementary Table A1 for a multivariate GLM on the various dependent variables considered in this study) and both "darkness" and "toxicity" select for (or impose constraints on) the same trait dynamics: reduced fecundity and increased offspring size.

### *Reduced resource availability as a selective factor?*

Caves are generally described as being resource–limited, because they lack photoautotroph primary production and thus rely on organic influx from surrounding surface habitats (Hüppop 2000; Poulson and Lavoie 2000). Sulfidic habitats, on the other hand, have been described to be nutrient–rich due to chemoautotroph primary production (Langecker et al. 1996). So how does this relate to the sulfidic Cueva del Azufre? Like other sulfidic habitats, the Cueva del Azufre is also resource–rich; however this does not necessarily mean that cave mollies have a surplus of resources to put into reproduction or growth. Detoxifying $H_2S$ is energetically costly (Sibly and Calow 1989), so that acquired resources are most likely immediately funneled into physiological detoxification of $H_2S$. Additionally, *P. mexicana* in sulfidic habitats rely on aquatic surface respiration (ASR) to exploit the more oxygenated (and thus less sulfidic) top–most layer of the water column (Plath et al. 2007*b*; Tobler et al. 2009*a*). In a recent study, we could demonstrate that time budgets of fish from sulfidic habitats are dominated by ASR, such that only a small proportion of their time can be spent feeding (for a more detailed discussion see Tobler 2008; Tobler et al. 2009*a*). These processes may actually lead to resource limitation in an otherwise resource–rich environment. Therefore, even if fat content did not differ between extreme and non–toxic surface habitats (but see Plath et al. 2005; Tobler et al. 2006, 2008*c*; Tobler 2008), the lower somatic lean weights found in extreme environments are still best explained by resource limitation.

Moreover, it should be noted that the absence of light in the two caves could make resource acquisition more difficult for cave mollies, regardless of the actual resource level in the respective cave. In fact, preliminary data suggests that cave mollies may not necessarily be more efficient in finding and acquiring food in darkness than surface mollies (R. Riesch, *unpublished data*).

### *Reduced fecundity*

Toxicity and darkness both lead to reduced fecundity, while at the same time offspring size increases. Reduced fecundity and increased offspring size in fish from the Cueva del Azufre clearly has a heritable component since it was found also in fish that were reared under common–garden conditions over several generations (Riesch et al. 2009*b*; Riesch et al. *in press*), which strongly suggests adaptive divergence as an evolutionary response to strong selection.

Reduced fecundity has been found in most comparisons of epigean and hypogean organisms (Christiansen 1965; Culver et al. 1995; Parzefall 2000; Hüppop 2000; Culver 2005), such as some cave fishes (e.g., cave amblyopsids [Poulson 1963]; a characid, *Astyanax mexicanus* [Hüppop and Wilkens 1991]; catfish, *Trichomycterus chaberti* [Pouilly and Miranda 2003]), and this was termed the ‘equilibrium strategy’ (Winemiller 1992). In our system, *P. mexicana* from cave habitats also exhibit the same life–history patterns with reduced fecundity and larger offspring, and fish with the lowest fecundity and largest offspring size come from the Cueva del Azufre, where both factors (“darkness” and “toxicity”) act in concert. However, it is interesting to note that the combination of darkness and toxicity results in lower fecundity (and, at least qualitatively, larger offspring size) than can be explained by a purely additive effect of both environmental stressors (as indicated by a significant interaction effect of “light condition by toxicity” for fecundity in Table A1).

### *Selection on increased offspring size and –autonomy*

Given that fish from the sulfidic surface habitats, as well as from the non–sulfidic and sulfidic caves are essentially energy–limited (regardless of the actual resource availability of the habitat), how exactly can reduced fecundity and large offspring size be adaptive?

Equilibrium strategists make a large investment into individual offspring to promote juvenile survivorship in crowded, resource–limited, and/or otherwise stressful environments (Winemiller 1992). In a recent paper, Bashey (2008) found supporting evidence for this in guppies, where larger offspring have a competitive advantage in crowded environments. Both the Luna Azufre and the Cueva del Azufre are characterized by areas of extremely high population densities (Tobler et al. 2006). In a recent study, Riesch et al. (2009*b*) argued that reduced fecundity is most likely caused by selection on offspring size in our study system, which would increase juvenile survivorship, for example, by decreasing the risk of starvation and increasing offspring mobility for more efficient foraging (Poulson and White 1969; Pouilly and Miranda 2003). Furthermore, intracohort cannibalism is widespread among poeciliids (e.g., *Gambusia affinis* [Dionne 1985; Meffe and Crump 1987]; *X. hellerii* [Jones et al. 2007]; *Poecilia latipinna* and *P. formosa* [Hubbs and Schlupp 2008]). In western mosquitofish (*G. affinis*), low food availability increased the rate of cannibalism (Dionne 1985), and bigger offspring are less likely to be preyed upon by conspecific adults in topminnows (*Poeciliopsis* spp. [Weeks and Gaggiotti 1993]) and mosquitofish (*Gambusia* spp. [Hubbs 1991, 1992]). Even though studies on cannibalism are as yet lacking in this system, it has long been proposed to be a major factor in the Cueva del Azufre, and bigger offspring are most likely less prone to being preyed upon by other adult *P. mexicana*.

Finally, increased offspring size leads to a decreased body surface: volume ratio, so less surface area per volume of body tissue will be exposed to hydrogen sulfide in toxic environments. Furthermore, larger offspring probably have lower metabolic rates and lower oxygen consumption per volume body tissue, which would be generally adaptive in resource–limited environments. Hence, larger offspring most likely perform ASR more efficiently.

### *General life-history evolution in caves*

In addition to increased offspring size and reduced fecundity, a variety of other life–history traits have been described to characterize cave organisms: reduced reproductive effort, delayed reproduction, reduced growth rates, decreased proportion of the population breeding at any given time, and increased life span, which also results in an increase in the maximum number of broods (Parzefall 2000; Culver 2005). Compared to their closest relatives, *P. mexicana* from southern Mexican non–toxic surface waters (SM), cave mollies from both caves did indeed have a lower proportion of breeding females at any given time (but see Riesch et al. 2009*b*). On the other hand, if one takes the northern Mexican populations into account ($NM_1$ and $NM_2$) this pattern did not hold.

Furthermore, reproductive allocation (RA, our measure of reproductive effort) did also not quite follow the predicted pattern: while RA was lower in mollies from the non–toxic Luna Azufre, it was actually highest in mollies from the toxic Cueva del Azufre. Toxicity, however, seems to select for increased reproductive allocation in *P. mexicana* (it is interesting to note that this is not the case in two other sulfur-endemic poeciliids, *Gambusia eurystoma* and *Poecilia sulphuraria*, nor in *Gambusia sexradiata* which sometimes occurs in sulphidic surface waters: Riesch et al. 2010); hence, it appears as if the toxicity in the Cueva del Azufre exerts stronger selection on increased reproductive allocation, ultimately offsetting the reduction of reproductive allocation induced by the absence of light, thus resulting in a higher reproductive allocation in the Cueva del Azufre than in either the non–toxic cave or the non–toxic surface habitats.

Finally, data on lifespan, lifetime reproductive effort, maximum number of broods, or age of first reproduction in the *P. mexicana* system are as yet limited. However, preliminary analysis from a common–garden rearing experiment seems to indicate that cave mollies from the Cueva del Azufre are indeed characterized by delayed maturity and reduced growth rates (R. Riesch*, unpublished data*). Therefore, if cave mollies also have a longer lifespan than surface mollies, lifetime reproductive effort and maximum brood number could indeed be larger in both cave molly populations (in particular in cave mollies from the Cueva del Azufre which are already characterized by increased reproductive allocation per clutch).

### *The role of predation in the cave molly system*

Traditionally, a variety of differences in poeciliid behavior, morphology, and life history have been attributed to differences in predatory regimes between populations (e.g., Reznick and Endler 1982; Langerhans et al. 2007). As we have demonstrated elsewhere, there are obvious differences in predator regimes between sulfidic and non-sulfidic habitats, as well as between surface and cave habitats: while fish communities (including piscivorous fishes) are usually complex in benign surface habitats, they are highly reduced in sulfidic and cave habitats with extremophile poeciliids being the only permanent piscine residents (Riesch et al. 2006, 2009*a*; Tobler et al. 2006, 2008*c*). However, this does not mean that extremophile poeciliids are safe from predation. On the contrary, the piscivorous bird community is essentially the same for both types of surface habitats and bird predation actually appears to be higher in sulfidic surface habitats (Riesch et al. 2009*a*). This is probably due to extremophile poeciliids having to stay close to the water surface to perform ASR (Plath et al. 2007*b*; Tobler et al. 2009*a*), which makes them relatively easy prey for piscivorous birds. The only known predators in the Cueva del Azufre, on the other hand, are giant water bugs (*Belostoma* sp.) and several species of large-bodied spiders (Tobler et al. 2007, 2008*b*; Tobler 2009; Horstkotte et al. in press). These cave predators are very abundant and they seem to exert high predation pressures on cave mollies (Tobler et al. 2007). Hence, cave mollies do not live in predator–free environments, as has been suggested for other cave fishes (Romero and Green 2005).

### *Maternal provisioning*

With the exception of Luna Azufre females, all populations had a matrotrophy index between 0.57 and 0.72, which according to this method would be interpreted as pure lecithotrophy. The embryo weight loss during development of 28 – 43 % was well within the range of dry weight loss in other oviparous fishes (25 – 55 %: Wourms et al. 1988). Luna Azufre females (MI = 0.86), however, clearly showed some degree of post–fertilization provisioning. This finding highlights the importance of intraspecific variation when interpreting maternal provisioning in poeciliids, and our results suggest that *P. mexicana* is at least capable of employing so–called ‘incipient matrotrophy’ or dual provisioning (Blackburn 1992; Marsh-Matthews et al. *in press*; Riesch et al. *in press*).

### *Divergent life–histories and their potential role for reproductive isolation*

We have shown that fish from extreme habitats have lower fecundity and larger offspring than fish from non–toxic surface habitats. Nonetheless, even among extreme habitats fecundity and offspring size vary greatly, with the extreme values being found in the Cueva del Azufre, where both environmental stressors (“darkness” and “toxicity”) work in unison. These patterns could be explained by two mechanisms that are not mutually exclusive. It could be possible that some of the observed life–history differences between (in particular) the ‘seasonless’ caves (e.g., Poulson and White 1969; Culver 2005; Tobler et al. 2006, 2008*a*) and the surface habitats are simply due to seasonal plastic variation in the latter (e.g., lack of seasonal cues for reproduction in the caves or plastic responses to seasonal fluctuations in the surface habitats: Chapman et al. 1991; Chapman and Chapman 1993; Culver 2005; Tobler et al. 2006, 2008*a*). The same holds true for the comparison of surface habitats across Mexico. In southern Mexico, we tried to counteract this by sampling during both the dry season (June) and the rainy season (January) in all habitats (both caves, the El Azufre and SM), but some of the observed variation may still be accounted for by plastic responses to seasonal differences.

However, with regard to fecundity and offspring size we have evidence that plasticity is only part of the explanation: in recent studies we found that reduced fecundity and increased offspring size in fish from the Cueva del Azufre are clearly heritable (Riesch et al. 2009*b*; Riesch et al. *in press*). Studies on cave Amblyopsidae have also demonstrated that cave–fish life histories are not simply constraints imposed by the environment, but are, at the very least, combinations of environmentally induced constraints and naturally selected evolutionary adaptations (review in Culver 2005). Hence, our results hint at adaptive trait divergence as another possible mechanism of ecological diversification, where the extent of reduction in fecundity and increase in offspring size is beneficial in the respective extreme habitat, but females will most likely be outcompeted in the 'wrong' habitat type. For example, dispersing individuals with low fecundity will be outcompeted in non–extreme habitats, where females produce far more offspring. Competition by better locally–adapted individuals (competitive exclusion) along with increased juvenile mortality (due to the 'wrong' offspring size in the 'wrong' habitat) most likely results in strongly decreased fitness of dispersing individuals in this system. This, however, has to be put into perspective: when *P. mexicana* initially invaded the novel extreme environments, ecological divergence most likely caused the original divergence in life–history evolution. Nonetheless, we propose that divergent life histories at this point in time in our study system act as an additional mechanism that—along with trophic (Tobler 2008), morphological (Tobler et al. 2008*a*, *c*) and behavioral divergence (e.g., Plath et al. 2003, 2005, 2007*b*)—effectively restricts gene flow through direct selection against 'migrants' (Plath et al. 2007*a*; Tobler et al. 2008*a*, 2009*a*). In summary, disruptive life–history trait evolution due to local adaptations to different habitat types provides another mechanistic link promoting ecological diversification, and ultimately parapatric speciation, in this system.

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**Table 1.** Descriptive statistics for life–history traits of female *Poecilia mexicana* from four different habitat types and three different drainages. SM, southern Mexican Río Grijalva/Usumacinta drainage; $NM_1$, northern Mexican Río Soto la Marina drainage; $NM_2$, northern Mexican Río Tamesí/Pánuco drainage; MI, Matrotrophy Index.

| Habitat type (Population) | *N*[a] | Mean SL[b] [mm] of reproducing females | Female Fat Content[b] [%] | Female Lean Weight[b] [g] | Fecundity[b,c] [# offspring] | RA[b,d] [%] | Estimated Embryo Dry Weight at Birth[e] [mg] | Embryo Fat Content[b] [%] | MI[f] |
|---|---|---|---|---|---|---|---|---|---|
| dark/toxic: | | | | | | | | | |
| (Cueva del Azufre) | 37/83 | 36.97±4.59 | 6.21±4.41 | 0.20±0.07 | 5.79±7.91 | 13.73±4.34 | 6.61 | 15.75±4.04 | 0.57 |
| dark/non-toxic: | | | | | | | | | |
| (Luna Azufre) | 31/47 | 31.26±3.57 | 8.52±4.99 | 0.13±0.04 | 12.89±8.80 | 9.93±3.37 | 4.24 | 14.12±2.94 | 0.86 |
| light/toxic: | | | | | | | | | |
| (El Azufre) | 27/71 | 31.44±4.40 | 2.74±2.15 | 0.17±0.09 | 14.67±8.63 | 12.29±4.15 | 3.75 | 18.22±4.76 | 0.71 |
| light/non-toxic: | | | | | | | | | |
| (SM) | 62/84 | 39.21±7.95 | 9.93±4.73 | 0.31±0.20 | 20.14±7.87 | 11.18±2.96 | 2.59 | 20.75±4.81 | 0.58 |
| ($NM_1$) | 20/50 | 50.60±4.36 | 6.68±4.32 | 0.71±0.18 | 9.96±9.08 | 12.30±3.87 | 3.54 | 18.58±2.24 | 0.63 |
| ($NM_2$) | 27/80 | 45.48±8.60 | 3.47±2.88 | 0.57±0.29 | 13.67±8.50 | 11.35±5.19 | 2.68 | 16.37±4.28 | 0.72 |

[a] numerator corresponds to females with developing young (=effective sample size); denominator equals the total number of dissected females.

[b] mean ± S.D.

[c] estimated marginal means ± S.D. from a GLM with SL as a covariate.

[d] Reproductive Allocation: Proportion of total dry weight which consists of developing embryos.

[e] estimated weight at stage "6 = neonate" after Reznick (1981) using the slope and constant from the regression between embryonic dry weight and stage of development.

[f] estimated dry weight at birth divided by dry weight at fertilization (using the slope and constant from the regression between embryonic dry weight and stage of development).

## FIGURE LEGENDS

**Figure 1.** Map of the collection sites in Mexico. Major drainages are underlined and major cities in grey. Sample sites are (1) & (2) Río Soto la Marina drainage ($NM_1$), (3) & (4) Río Tamesí/Pánuco drainage ($NM_2$), and (5) the southern Mexican Río Grijalva/Usumacinta drainage (SM) including the cave plateau.

**Figure 2.** (*a*) Discriminant function analysis for separation of drainages/extreme habitats based on female life–history traits while controlling for female size (SL) and embryo stage. (△) SM, (◇) $NM_1$, (☐) $NM_2$ (all light/non-toxic), (●) El Azufre (light/toxic), (▲) Luna Azufre (dark/non-toxic), and (●) Cueva del Azufre (dark/toxic). (*b*) DFA for separation among habitat types based on female life–histories while controlling for female size (SL) and embryo stage. Group centroids ± SDs.

**Figure 3.** Principal component analyses based on female life–history traits. The non–toxic surface habitats were SM, $NM_1$, and $NM_2$. These were contrasted against the toxic cave (Cueva del Azufre), the toxic surface habitat (El Azufre), and the non–toxic cave (Luna Azufre).

**Figure 4.** Scatter plots of mean embryo dry weight [mg] versus stage of development of *Poecilia mexicana* for four different habitat types. Stages are determined on a progressive scale with the earliest (5) being the neurula stage and the oldest (50) being equivalent to ready–to–be–born embryos (after Haynes 1995, Reznick et al. 2002).

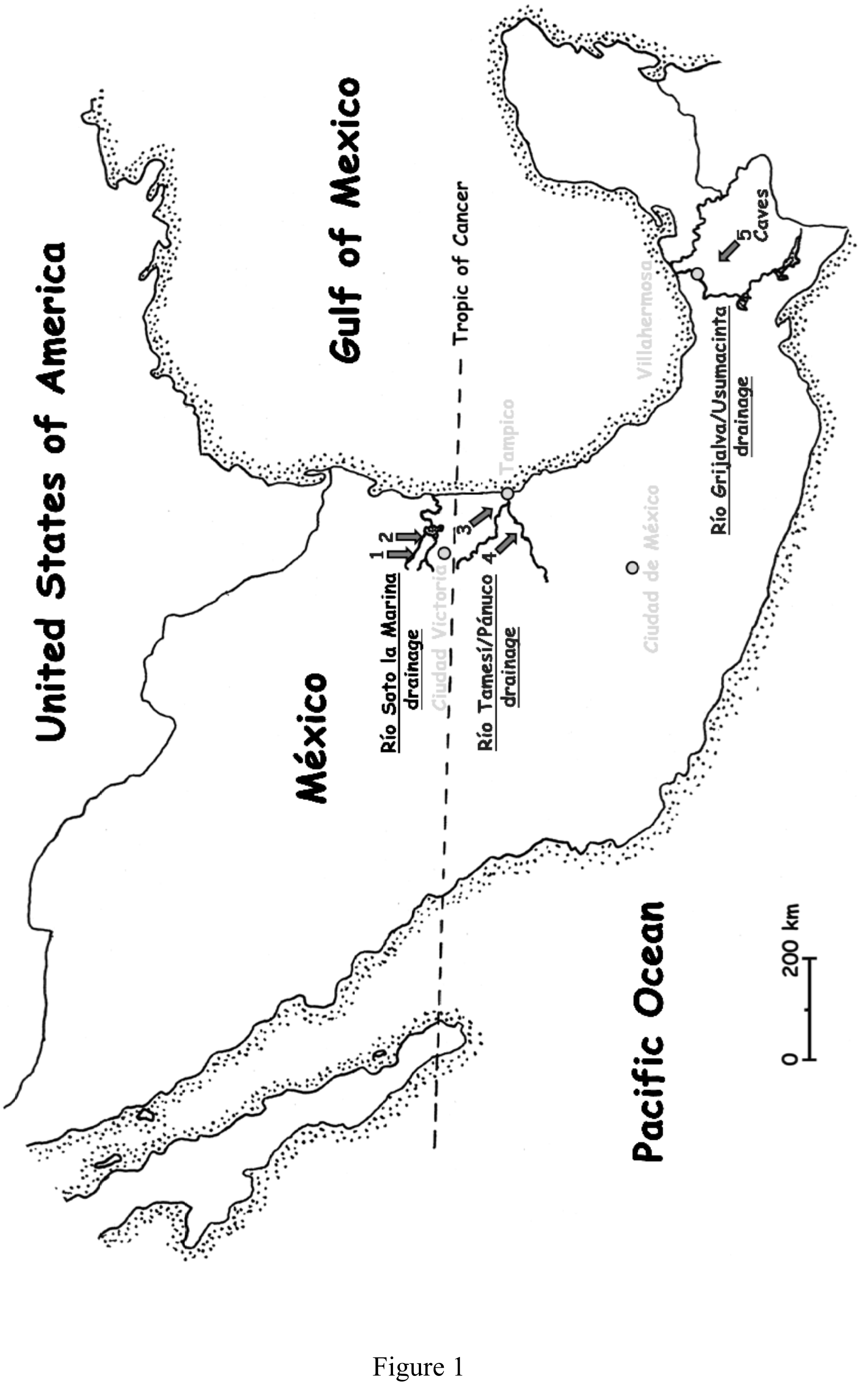
United States of America
Gulf of Mexico
Tropic of Cancer
México
Río Soto la Marina drainage
Ciudad Victoria
1
2
3
4
Río Tamesí/Pánuco drainage
Tampico
Ciudad de México
Villahermosa
Río Grijalva/Usumacinta drainage
5
Caves
Pacific Ocean
0
200 km

Figure 1

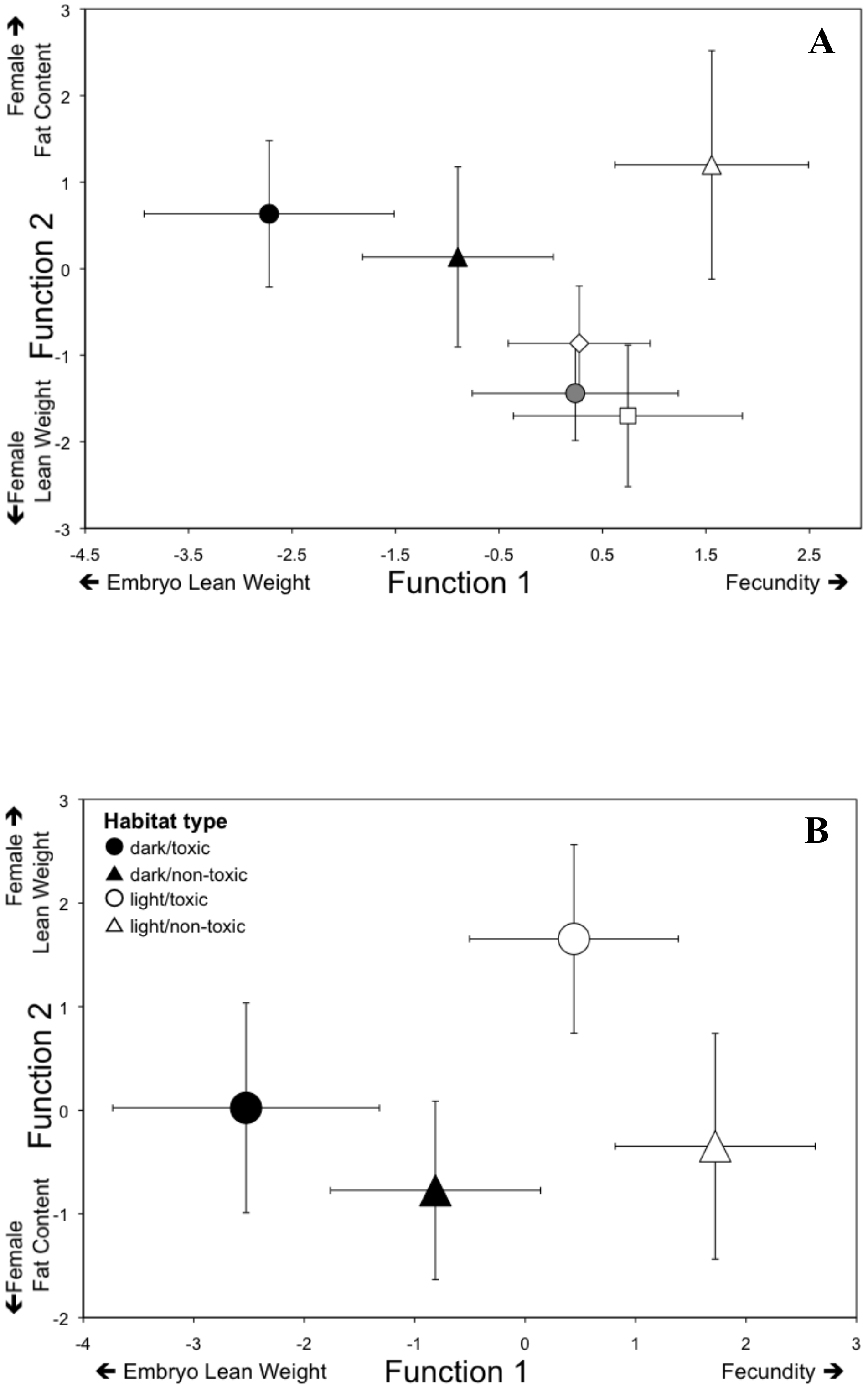
A
Female Fat Content ↑
Function 2
↓Female Lean Weight
3
2
1
0
-1
-2
-3
-4.5
-3.5
-2.5
-1.5
-0.5
0.5
1.5
2.5
← Embryo Lean Weight
Function 1
Fecundity →
B
Habitat type
● dark/toxic
▲ dark/non-toxic
○ light/toxic
△ light/non-toxic
Female Lean Weight ↑
Function 2
↓Female Fat Content
3
2
1
0
-1
-2
-4
-3
-2
-1
0
1
2
3
← Embryo Lean Weight
Function 1
Fecundity →

Figure 2

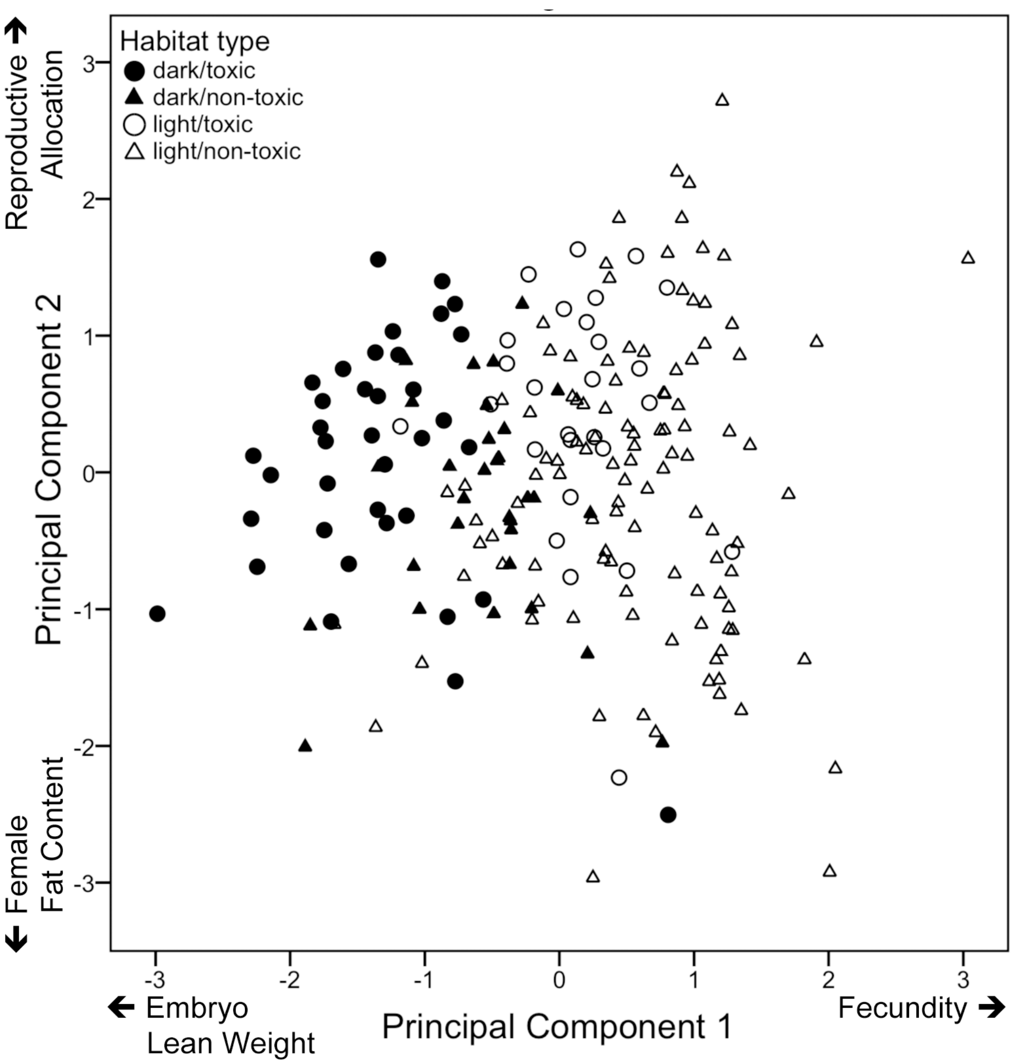
Habitat type
● dark/toxic
▲ dark/non-toxic
○ light/toxic
△ light/non-toxic
Reproductive Allocation
Principal Component 2
Female Fat Content
3
2
1
0
-1
-2
-3
-3
-2
-1
0
1
2
3
Embryo Lean Weight
Principal Component 1
Fecundity

Figure 3

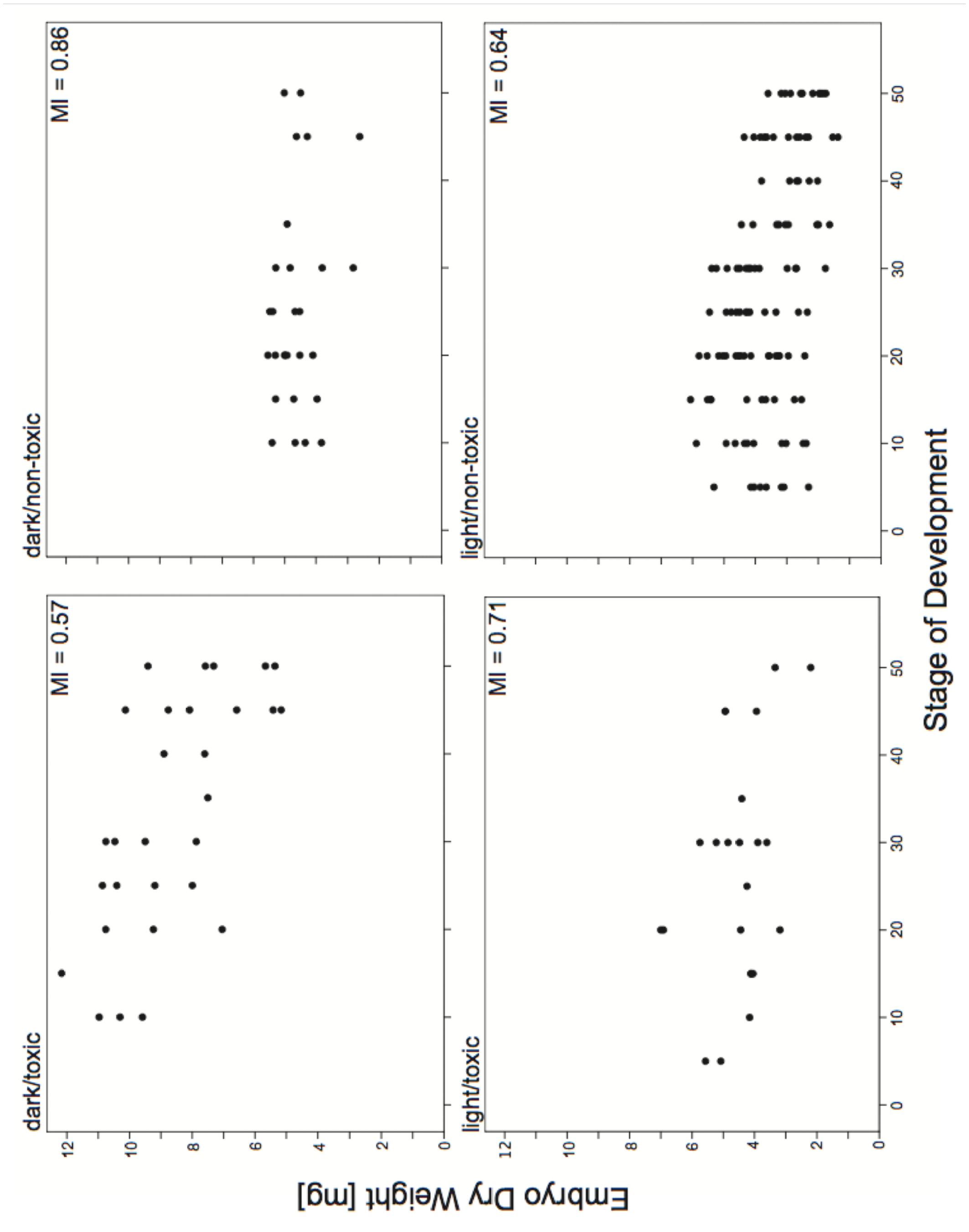
dark/toxic
MI = 0.57
dark/non-toxic
MI = 0.86
light/toxic
MI = 0.71
light/non-toxic
MI = 0.64
0
2
4
6
8
10
12
0
10
20
30
40
50
Embryo Dry Weight [mg]
Stage of Development

Figure 4

# CHAPTER 3

## CONVERGENT LIFE-HISTORY SHIFTS: TOXIC ENVIRONMENTS RESULT IN BIG BABIES IN TWO CLADES OF POECILIIDS

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Keywords: Ecological speciation, divergent natural selection, extremophile fish, *Gambusia*, hydrogen sulfide, offspring size, *Poecilia*

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# ABSTRACT

The majority of studies on ecological speciation in animals have investigated divergence caused by biotic factors like divergent food sources or predatory regimes. Here we examined a system where ecological speciation can clearly be ascribed to abiotic environmental gradients of naturally occurring toxic hydrogen sulfide ($H_2S$). In southern Mexico, two genera of livebearing fishes (Poeciliidae: *Poecilia* and *Gambusia*) thrive in various watercourses with different concentrations of $H_2S$. Previous studies have revealed pronounced genetic differentiation between different locally adapted populations in one species (*P. mexicana*), pointing towards incipient speciation. In the present study, we examined female reproductive life-history traits in two species-pairs: *G. sexradiata* (from a non-sulfidic and a sulfidic habitat) and *G. eurystoma* (sulfide-endemic) as well as *P. mexicana* (non-sulfidic and sulfidic) and *P. sulphuraria* (sulfide-endemic). We found convergent divergence of life-history traits in response to sulfide; most prominently, extremophile poeciliids exhibit drastically increased offspring size coupled with reduced fecundity. Furthermore, within each genus, this trend increased with increasing sulfide concentrations and was most pronounced in the two endemic sulfur-adapted species. We discuss the adaptive significance of large offspring size in toxic environments, and propose that divergent life–history evolution may promote further ecological divergence through isolation-by-adaptation.

# INTRODUCTION

## *Ecological speciation*

Ecological speciation describes the process during which reproductive isolation evolves as the result of ecologically-based divergent natural selection (Schluter 2000, 2001; Rundle and Nosil 2005). Evidence for ecological speciation comes from theoretical considerations, empirical studies in natural systems, and from laboratory evolution experiments (reviewed by Rundle and Nosil 2005; Hendry et al. 2007; Nosil et al. 2009a, 2009b). Most studies on ecological speciation in animals focused on biotic selective agents like divergent food types (e.g., Hatfield and Schluter 1999; Rundle 2002), differences in predation risk (e.g., Nosil and Crespi 2006; Langerhans et al. 2007), or the degree of parasitism (e.g., Anderson et al. 1990; Nyman et al. 2007). While the literature on ecological speciation due to abiotic selective agents and stressors is quite extensive for plants (e.g., MacNair and Christie 1983; Rajakaruna et al. 2003; Antonovics 2006; Hays 2007; Jiménez-Ambriz et al. 2007), only relatively few studies to date investigated ecological speciation along abiotic environmental gradients in animals (e.g., Fuller et al. 2007). Even though stressful environments have long been known to be associated with bouts of directional selection (Hoffmann and Parson 1997), the concept of stress and the maintenance of homeostasis through adaptation (Romero 2004) is often ignored in the study of speciation in animals and more emphasis is put on its role in population decline and extinction (Lexer and Fay 2005).

Furthermore, what exactly is the role of divergent life-history trait evolution during ecological speciation events? In a recent study (Riesch et al. in press) we have suggested that, after an initial phase of divergent natural selection due to local environmental differences, divergent life-history traits at a later phase promote reproductive isolation if individuals and their offspring have reduced fitness when migrating/dispersing into a habitat to which they are not locally adapted.

### *Hydrogen sulfide as a selective agent*

Hydrogen sulfide ($H_2S$) is a widespread natural toxicant, and its patchy occurrence often creates environmental gradients in aquatic ecosystems (both horizontally and vertically), e.g. at deep-sea hydrothermal vents, in coastal salt marshes, or mudflats (Bagarinao 1992; Grieshaber and Völkel 1998). Hydrogen sulfide is acutely toxic to most metazoans and leads to extreme hypoxia in the water. Even at sub-lethal concentrations it has several adverse effects on non-adapted fishes, among them reduced egg production, smaller-sized larvae, and decreased gonadosomatic index (reviewed by Bagarinao 1992). Organisms able to cope with persistently high concentrations of $H_2S$ usually exhibit various specific adaptations (e.g., larger heads and bigger gill surface area: Tobler et al. 2008a; for reviews see Bagarinao 1992; Grieshaber and Völkel 1998). Hence, $H_2S$ is a source of strong divergent selection between locally adapted populations/species and populations/species that cannot tolerate it and may drive speciation events (Tobler and Plath in press; Plath and Tobler in press).

In Tabasco, southern Mexico, livebearing fishes (Poeciliidae) inhabit various freshwater ecosystems in the Río Grijalva/Usumacinta drainage, some of which are characterized by the presence of naturally occurring $H_2S$ (Tobler et al. 2006; Tobler et al. 2008b), which appears to be of volcanic origin (Rosales-Lagarde et al. 2008). The presence of $H_2S$ represents a constant stressor in these environments, even though toxicity levels fluctuate in time and space within the habitats (e.g., Tobler et al. 2008a, 2008b). Two species in particular, *Gambusia sexradiata* and *Poecilia mexicana*, inhabit various non-sulfidic habitats throughout the area, but also thrive in several sulfidic habitats (Tobler et al. 2006, 2008b). Nested within the distribution of these two poeciliids is the geographic distribution of two closely-related species (*G. eurystoma* and *P. sulphuraria*), which are endemic to a small sulfidic stream at the Baños del Azufre (Alvarez del Villar 1948; Miller 1975; Tobler et al. 2008b). Even though the divergent habitats are not physically isolated, toxicity apparently creates an island-like situation in these systems, effectively preventing immigration by non-adapted individuals from the surrounding benign habitats. In the case of *P. mexicana*, we could indeed demonstrate lack of gene flow between populations from adjacent sulfidic and non-sulfidic habitats (Plath et al. 2007a), along with divergent behavioral (Plath et al. 2003, 2004), morphological (Tobler et al. 2008a), trophic (Tobler 2008), and life-history trait evolution (Riesch et al. 2009b, in press).

Altogether, our system is of particular interest to study ecological speciation, because both species-pairs (*G. sexradiata*-*G. eurystoma*, and *P. mexicana*-*P. sulphuraria*) provide us with phylogenetically independent replication (molecular phylogenies for both genera: Lydeard et al. 1995; Ptacek and Breden 1998), with the *G. sexradiata* and *P. mexicana* phenotypes as the most likely ancestral character states from which the endemic sulfur-adapted forms independently evolved. Hence, our system allows for a comparative approach, where we find populations resembling the potential ancestral phenotypes (*G. sexradiata* and *P. mexicana* from benign habitats), two forms that have already reached full reproductive isolation (*G. eurystoma* and *P. sulphuraria*, respectively), and intermediate populations probably undergoing ecological speciation at present (*G. sexradiata* and *P. mexicana* from sulfidic habitats; for *P. mexicana* see Plath et al. 2007a; Tobler et al. 2008a, 2009b; Riesch et al. in press). Furthermore, these species live in—and apparently can tolerate—different concentrations of hydrogen sulfide (e.g., Plath et al. 2007b; Tobler et al. 2008b), making this system ideal to study divergence along an environmental gradient.

In the present study, we investigated life-history divergence in response to varying levels of $H_2S$. Based on reports on life-history traits in other fishes exposed to hydrogen sulfide (where $H_2S$ constrains reproduction rather than selecting for specific adaptations), we predicted to find two trends within each species-pair: (1) as a direct result of exposure to $H_2S$ fishes from sulfidic habitats should have lower body condition (measured as percent body fat), lower fecundity, smaller offspring, and lower reproductive allocation (percent body weight consisting of offspring), and (2), because *G. eurystoma* and *P. sulphuraria* are clearly well-adapted to high concentrations of $H_2S$ (i.e., being sulfur-endemic), we hypothesize that some of these traits should show some kind of rebound-effect indicating evolved mechanisms to cope with the toxin.

# METHODS

## *Field collections*

All field collections were made in Tabasco, southern Mexico, and all fish were field preserved in 10% formaldehyde. Individual *Poecilia mexicana* were collected in June 2007 and January 2008, *Gambusia sexradiata*, *G. eurystoma,* and *P. sulphuraria* were collected in August 2008, and January 2009. The data for *P. mexicana* females were re-analyzed from a previous study (Riesch et al. in press). For a detailed sampling list, please refer to the Appendix; however, note that *P. mexicana* from non-sulfidic habitats were collected at two separate sites, which are both part of the Río Oxolotán sub-drainage and will therefore henceforth only be referred to as Río Oxolotán. We were only able to acquire restricted permits for sampling of the endemic species *G. eurystoma and P. sulphuraria*. This forced us to specifically collect only reproductive females from both species, and for the same reason we were so far unable to conduct common-garden rearing experiments.

To determine $H_2S$ concentrations, we collected two water samples at all collection sites. Using a syringe, one milliliter of water was injected into a vial containing 2 ml of zinc acetate (0.12 M with 0.5 ml NaOH 1.5 M) and photometric measurements were conducted according to Cline (1969).

## *Life–history analyses*

Following the protocol of Reznick and Endler (1982), all preserved fish were weighed and measured for standard length (to the nearest milligram and millimeter, respectively). The reproductive tissue and, if present, all developing offspring were removed. Offspring were counted and their stage of development determined (Reznick and Endler 1982; Haynes 1995). Somatic tissues, reproductive tissues and embryos were then dried for 10 days at 40°C and re-weighed. To assess female and embryo condition, somatic tissues and embryos were rinsed six times for at least six hours in petroleum ether to extract soluble nonstructural fats (Heulett et al. 1995), and were then re-dried and re-weighed. Furthermore, we calculated the reproductive allocation (RA) by dividing offspring weight by the sum of offspring weight plus somatic dry weight (Reznick and Endler 1982: here called reproductive allotment).

### *General linear model (GLM)*

We tested for differences in female life-histories between habitats and genera by means of a multivariate GLM (MANCOVA) with “female lean weight [g]”, “female fat content [%]”, “fecundity”, “reproductive allocation [%]”, “embryo lean weight [mg]”, and “embryo fat content [%]” as dependent variables, “female standard length [mm]” and “embryo stage” as covariates, and “genus (two levels: *Gambusia* vs. *Poecilia*)” and “toxicity-exposure (three levels: non–toxic vs. incipient-toxic vs. endemic-toxic)” as independent variables.

All dependent variables and covariates were either log–transformed or arcsine–transformed (in case of percentages) to accommodate for potential non–linear relationships between the variables (Quinn and Keough 2002). Our original model incorporated all possible interactions of all covariates and factors, but all non-significant interactions ($F \leq 1.750$, $P \geq 0.055$) were removed from the final model in a step-wise process.

### *Discriminant function analyses (DFAs)*

To provide an intuitive metric with respect to the magnitude of life-history divergence we conducted several discriminant function analyses (DFA). We used a jackknife (leave-one-out) sampling scheme as a cross-validation technique (i.e., each case is classified by the functions derived from all cases other than that case). For each DFA, *a priori* probabilities were calculated based on group-sizes and an overall classification success was calculated.

The first DFA tested for discrimination along the factors ‘toxicity-exposure’ and “genus”. The grouping variables were “*Poecilia*/non-toxic”, “*Poecilia*/incipient-toxic”, “*Poecilia*/endemic-toxic”, “*Gambusia*/non-toxic”, “*Gambusia*/incipient-toxic”, and “*Gambusia*/endemic-toxic”. To accommodate for the effects of female standard length (SL) and embryo stage on various life-history traits, we used residuals from a preparatory multivariate GLM as dependent variables in which “SL” and “embryo stage” were included as covariates. The dependent variables for the GLM (and their residuals for the DFA) were “female lean weight [g]”, “female fat content [%]”, “fecundity [number of offspring]”, “reproductive allocation [%]”, “embryo lean weight [mg]”, and “embryo fat content [%]”. Again, all dependent variables and covariates were either log-transformed or arcsine-transformed.

In a second DFA, we tested for classification success based on “toxicity-exposure” alone. The same dependent variables (as residuals) were used as in the previous analysis; however, this time the grouping variables were “non-toxic”, “incipient-toxic”, and “endemic-toxic”.

Finally, in a third DFA, we tested for classification success based on "toxicity"-effects alone. Here, the grouping variables were "toxic" and "non-toxic".

### *Principal components analysis (PCA)*

We conducted PCA to test for classification based on life-history characters without defining groups prior to analysis. The dependent variables ("female lean weight [g]", "female fat content [%]", "fecundity [number of offspring]", "reproductive allocation [%]", "embryo lean weight [mg]", and "embryo fat content [%]") were again the residuals from a preparatory multivariate GLM with the covariates "SL" and "embryo stage".

All statistical analyses were conducted using SPSS 16.0.2 for Mac (SPSS Inc., 2008).

## RESULTS

### *Descriptive statistics and MANCOVA*

Descriptive statistics for various life-history parameters are given in Table 1: Female fat content dropped dramatically from *Poecilia*/non-toxic to *Poecilia*/incipient-toxic but was high again in *Poecilia*/toxic-endemic (Table 1). In *Gambusia*, however, female fat content was highest in *Gambusia*/non-toxic and lowest in *Gambusia*/endemic-toxic (Table 1).

Mean size-corrected fecundity showed the predicted pattern of lower fecundity in sulfidic habitats within both genera (Table 1). Reproductive allocation remained relatively constant in *Poecilia* from different habitats, but decreased considerably in sulfur *Gambusia* (Table 1).

Finally, embryo dry weight did not follow the predicted pattern, since embryos from sulfidic habitats were always larger than their counterparts from benign habitats (Table 1; Fig. 1). In fact, it appears as if the relationship between embryo dry weight and hydrogen sulfide concentration is non-linear and asymptotic (at least for *Poecilia*, which tolerates a broader range of $H_2S$ concentrations): low concentrations of hydrogen sulfide already lead to a big increase in embryo size, and embryo size soon approaches a maximum (Fig. 1).

As predicted both covariates in the MANCOVA had a significant influence on female life-history traits (e.g., larger females had a higher fecundity), even though the effect of embryo stage on female life histories was relatively weak (partial variance explained: 14.0%; Table 2). Furthermore, there were significant effects of “genus”, “toxicity-exposure”, and “genus by toxicity-exposure” on female life-history traits (Table 2). This indicates historical divergence in life histories (“genus” effect) between the two clades of poeciliid fishes, as well as some shared responses to sulfur (“toxicity” effect). However, the significant interaction of “genus by toxicity-exposure” indicates that besides some shared life-history shifts, each genus also exhibits some unique responses to $H_2S$ (see above; Table 1).

### *DFAs and PCA*

The first DFA based on “genera/toxicity-exposure” classified 72.2 % of the females into the correct group (Fig. 2; Supplementary Table 3). The second DFA was based on “toxicity-exposure” alone and classified 82.5 % of the females into the correct group (Supplementary Table 4, Supplementary Fig. 1), and the DFA on “toxicity” was to

91.0 % successful (Supplementary Table 4). The variables with the most discriminatory power in all DFAs were fecundity and embryo lean weight (Supplementary Table 3 and 4).

This strong differentiation between toxic and non-toxic habitats was clearly reflected in the PCA (Fig. 3; Supplementary Table 5), the first two components of which alone accounted for 61% of the variance. Like in the DFAs, the two life-history variables that accounted for most of the variation along principal component 1 were embryo lean weight and fecundity (Fig. 3; Supplementary Table 5).

## DISCUSSION

In the present study we investigated life-history traits in females of four poeciliid species (*P. mexicana, P. sulphuraria*, *G. sexradiata*, and *G. eurystoma*) from six different sites that differed in the degree of hydrogen-sulfide toxicity, and we asked whether certain life-history traits are commonly selected for/constrained by hydrogen sulfide regardless of species/genus. We found that several life-history traits showed similar trends between species from the same habitat type. Most notably, extremophile poeciliids shared reduced fecundity but drastically increased offspring size. At the same time we found some unique responses to toxicity based on the genus-specific life-history differences between *Gambusia* and *Poecilia* (e.g., Reznick and Miles 1989) that served as respective starting points for the shifts observed in toxicity. We have to point out, however, that some of the observed differences due to the effects of “genus” and “genus by toxicity-exposure” could simply be artifacts of our sampling scheme and could therefore also represent some seasonal and/or yearly variation.

The pattern we found here, with strong similarities between life-histories of *P. sulphuraria* and *P. mexicana* from sulfidic habitats, is consistent with our interpretation from other studies in which we proposed that *P. mexicana* from the sulfidic El Azufre are currently undergoing ecological speciation (Plath et al. 2007a; Tobler et al. 2008a, 2009b; Riesch et al. in press). In this particular system, gene flow between adjoining sulfidic-benign *P. mexicana* populations is very low despite the absence of physical barriers (Plath et al. 2007a; Tobler et al. 2008a), and there is pronounced divergence between populations in trophic ecology (Tobler 2008), behavior (Plath et al. 2007b), and morphology (Tobler et al. 2008a). The two sulfide-endemic species (*G. eurystoma* and *P. sulphuraria*) clearly represent two possible endpoints of ecological speciation in response to $H_2S$, and *P. mexicana* from the sulfidic El Azufre appears to be strongly enroute to a similar state.

Do the divergent life-history traits reported here (most prominently the combination of low fecundity and large offspring size) represent adaptations or are they simply constrained by $H_2S$? Even though we are as yet lacking comprehensive common garden rearing-experiments in this system, several facts strongly support our interpretation that several of these traits are indeed (heritable) adaptations: $H_2S$ has been documented to have a variety of adverse effects on fishes, including the occurrence of necrosis, inhibited spawning behavior, reduced egg production, reduced egg survival, smaller-sized larvae with high rates of deformities, and decreased gonadosomatic index (GSI) (reviewed by Bagariano 1992). On initial inspection, some of our results appear to point in this direction, namely reduced egg production (i.e., fecundity); still, female reproductive allocation did not differ to the same extent among *Poecilia* populations. Also, in *Gambusia*, reproductive allocation was highest in non-sulfidic habitats, lowest in *G. sexradiata* from slightly sulfidic waters, and showed the predicted rebound-effect in *G. eurystoma*. Moreover, within the genus *Poecilia* fat content was actually the highest in the endemic sulfur form, so life-history trait differences among populations were clearly not just due to poor body condition. Of particular interest is that extremophile poeciliids actually produce larger, not smaller offspring than their counterparts from benign habitats, with the largest offspring found in the two endemic sulfur species. Heritability of divergent life history traits has been proven so far only for *P. mexicana* inhabiting the sulfidic Cueva del Azufre (the subterranean source region of El Azufre; Riesch et al. 2009b; R. Riesch, M. Plath, I. Schlupp and E. Marsh-Matthews, unpublished manuscript), but seems likely also for the other forms investigated here. In particular, *P. sulphuraria* and *G. eurystoma* of wild-caught and first-generation laboratory stocks raised under benign (non-toxic) conditions still produce considerably larger and fewer offspring than *P. mexicana* or *G. sexradiata* raised under the same common garden conditions (R. Riesch, pers. observation).

If some of the observed life-history traits are in fact adaptive, what then is their adaptive significance? We propose several, not mutually exclusive explanations:

(1) In recent studies on another livebearing fish, the guppy *P. reticulata*, Bashey (2006, 2008) could demonstrate experimentally that increased competition (i.e. high population densities) selects for increased offspring size, as larger offspring have a competitive advantage (see also Brockelman 1975; Sibly and Calow 1983; Parker and Begon 1986). A similar scenario may accrue for the sulfidic systems

described here. Extremophile poeciliids from sulfidic habitats exhibit aquatic surface respiration (ASR) to cope with the adverse effects of toxic hydrogen sulfide (Plath et al. 2007b; Tobler et al. 2009a). During ASR they exploit the more oxygenated (and thus less sulfidic) air-water interface (Plath et al. 2007b). A recent time-budget analysis has shown that extremophile *Poecilia* spp. spend up to 84% of their time engaged in ASR (Tobler et al. 2009a). As a result, extremophile fish tend to cluster together in large groups at the water surface (authors, pers. observation), leading to very high local population densities.

(2) In extremophile poeciliids $H_2S$ has been shown to select for larger gill surface area, larger heads, and wider mouths (Tobler et al. 2008a; Fontanier and Tobler 2009). All of these adaptations are probably directly enhancing ASR-efficiency. Future experiments will need to determine whether larger offspring indeed perform ASR more efficiently.

(3) Increased offspring size leads to a decreased body surface: volume ratio, so less surface area per volume of body tissue will be exposed to the toxin. Furthermore, larger offspring most likely have lower metabolic rates and lower oxygen consumption than smaller offspring.

(4) Inter-cohort cannibalism is wide-spread among poeciliids (e.g., Meffe and Snelson 1989), and indeed, increased offspring size effectively limits the extent of cannibalism in topminnows, *Poeciliopsis* spp. (Weeks and Gaggiotti 1993). Furthermore, in the highly cannibalistic genus *Gambusia*, cannibalism is especially prevalent in species with small young (e.g., *G. affinis*, *G. speciosa*, and *G. geiseri*: mean weight ca. 1.75 mg), while it is less extensive in species with larger young (e.g.,

*G. nobilis*, *G. heterochir*, and *G. gaigei*: mean weight ca. 3.50 mg; Hubbs 1991, 1992). [As a note of caution, Hubbs (1992) did not explain whether the weights he presented were dry weights or wet weights; however, given a later paper (Hubbs 1995), in which he provides different values as wet weights for these species, we are inclined to think that the values provided are dry weights.] Although most of these findings come from lab experiments, there is evidence for cannibalism in the field (e.g., Meffe and Snelson 1989; Nesbit and Meffe 1993). Hence, the fact that *G. eurystoma* had the biggest offspring even though *P. sulphuraria* actually inhabit the most toxic waters, could be explained by differences in cannibalism rates between these two species.

The observed reduction in fecundity among fishes from sulfidic habitats, on the other hand, could indeed be simply constrained by the adverse effects of $H_2S$ as described for other fishes (Bagarinao 1992). However, as we argued above, increased offspring size is probably under strong directional selection, which simply constrains the maximum number of offspring in females from sulfur populations (Smith and Fretwell 1974). A direct adaptive benefit of low fecundity in extremophile poeciliids seems rather unlikely.

### *Predation in sulfidic habitats*

Traditionally, a variety of differences in poeciliid behavior, morphology, and life-history have been attributed to differences in predatory regimes between populations (e.g., Reznick and Endler 1982; Langerhans et al. 2007). As we have demonstrated elsewhere, there are obvious differences in predator regimes between sulfidic and non-sulfidic habitats: while fish communities (including piscivorous fishes) are usually complex in benign habitats, they are highly reduced in sulfidic habitats with extremophile poeciliids being the only permanent piscine residents (Tobler et al. 2006, 2008b; Riesch et al. 2006, 2009a). However, this does not mean that extremophile poeciliids are safe from predation. On the contrary, the piscivorous bird community is essentially the same for both habitat types and bird predation actually appears to be higher in sulfidic habitats (Riesch et al. 2009a). This is probably due to extremophile poeciliids having to stay close to the water surface to perform ASR (Plath et al. 2007b; Tobler et al. 2009a), which makes them relatively easy prey for piscivorous birds. Furthermore, in the guppy, predation is usually a proxy for a whole suite of correlated environmental differences (stream size, canopy cover, productivity, water temperatures, etc.: Endler 1995; Reznick et al. 2002). Hence, even though lab experiments have proven that predation alone can produce the observed shifts (e.g., Dzikowski et al. 2004; Gosline and Rodd 2008), in natural populations a variety of other factors are correlated with predation and are predicted to produce the same life-history patterns. Obviously, the same holds true for our system: a variety of divergent ecological factors is correlated with toxic $H_2S$ (see above). However, in our system all other (non-direct $H_2S$) differences between divergent environments are themselves again directly caused by $H_2S$. So, even though toxicity is not necessarily the only <u>direct</u> factor driving some of the observed shifts, it is at least the overarching <u>indirect</u> factor ultimately behind it all.

### *Conclusions*

We were able to demonstrate convergent shifts of life-history traits in extremophile poeciliids inhabiting sulfidic habitats. We conclude that the most likely scenario is that the original colonization of the divergent habitat types drove the life-history shifts we report here; however, we argue that at the present stage divergent life histories further facilitate reproductive isolation by imposing strong selection on dispersing/migrating individuals which could be maladapted to neighboring environments they disperse into.

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**Table 1.** Descriptive statistics for life–history traits of females from two genera (*Poecilia* and *Gambusia*) from three levels of evolutionary exposure to toxicity.

| Genus | Species (Habitat) | *N*[a] | Mean SL[b] [mm] of reproducing females | Female Fat Content[b] [%] | Female Lean Weight[b] [g] | Fecundity[b,c] [# offspring] | RA[b,d] [%] | Embryo Dry Weight[b,e] [mg] | Embryo Fat Content[b] [%] |
|---|---|---|---|---|---|---|---|---|---|
| *Poecilia* | *P. mexicana* (non-toxic) | 62/84 | 39.21±7.95 | 9.93±4.73 | 0.31±0.20 | 20.87±8.03 | 11.18±2.96 | 3.38±1.01 | 20.75±4.81 |
| | *P. mexicana* (incipient-toxic) | 27/71 | 31.44±4.40 | 2.74±2.15 | 0.17±0.09 | 5.70±2.08 | 12.29±4.15 | 4.50±1.00 | 18.22±4.76 |
| | *P. sulphuraria* (endemic-toxic) | 30/30* | 26.40±2.46 | 10.96±5.12 | 0.10±0.04 | 2.83±0.93 | 10.12±3.26 | 4.61±1.02 | 17.82±3.16 |
| *Gambusia* | *G. sexradiata* (non-toxic) | 47/53 | 24.19±3.60 | 10.42±4.04 | 0.08±0.04 | 17.34±5.69 | 23.03±5.84 | 1.40±1.08 | 16.78±2.44 |
| | *G. sexradiata* (incipient-toxic) | 19/40 | 29.00±2.26 | 7.96±2.35 | 0.15±0.04 | 9.32±3.57 | 10.46±4.42 | 1.86±1.00 | 17.70±3.65 |
| | *G. eurystoma* (endemic-toxic) | 27/27* | 23.63±2.20 | 4.59±2.60 | 0.07±0.02 | 2.19±0.99 | 15.63±4.61 | 6.45±1.01 | 17.23±2.52 |

[a] numerator corresponds to females with developing young (=effective sample size); denominator equals the total number of collected and dissected females.

[b] mean ± S.D.

[c] estimated marginal means ± S.D. from a GLM with SL as a covariate.

[d] Proportion of total dry weight which consists of developing embryos.

[e] estimated marginal means ± S.D. from a GLM with stage of development as a covariate.

* to accommodate for the limited permit we specifically collected pregnant females for these populations.

**Table 2.** Multivariate analysis of covariance (MANCOVA) examining female life-histories of four different species of poeciliids from two different genera and three levels of evolutionary exposure to toxicity. SL = standard length. All dependent variables and covariates were log–transformed (or arcsine–transformed if percentages) to accommodate for a potential non–linear relationship between the variables.

| Factor | *F* | *df* | *P* | Partial variance explained [%] |
|---|---|---|---|---|
| SL [mm] | 577.408 | 6, 199 | <0.001 | 94.6 |
| Embryo Stage | 5.408 | 6, 199 | <0.001 | 14.0 |
| Genus | 23.654 | 6, 199 | <0.001 | 41.6 |
| Toxicity-exposure | 57.234 | 12, 398 | <0.001 | 63.3 |
| Genus x Toxicity-exposure | 24.872 | 12, 398 | <0.001 | 42.9 |

*F*-ratios were approximated using Wilks' $\lambda$-values. Partial variance explained was estimated using Wilks's partial $\eta^2$.

## FIGURE LEGENDS

**Figure 1.** Relationship between sulfide concentrations and embryo size. *White square*, *Poecilia*/nontoxic; *white circle*, *Gambusia*/nontoxic; *light grey square*, *Poecilia*/incipient toxic; *light grey circle* *Gambusia*/incipient toxic; *dark grey square*, *Poecilia*/endemic toxic; and *dark grey circle*, *Gambusia*/endemic toxic.

**Figure 2.** Group centroids ± SDs of discriminant function analyses based on separation of female life-history traits based on toxicity-exposure and genus while controlling for female size (SL) and embryo stage. *White square*, *Poecilia*/nontoxic; *white circle*, *Gambusia*/nontoxic; *light grey square*, *Poecilia*/incipient toxic; *light grey circle*, *Gambusia*/incipient toxic; *dark grey square*, *Poecilia*/endemic toxic; and *dark grey circle*, *Gambusia*/endemic toxic.

**Figure 3.** Principal component analysis based on female life-history traits. *White squares*, *Poecilia*/nontoxic; *white circles*, *Gambusia*/nontoxic; *light grey squares*, *Poecilia*/incipient toxic; *light grey circles*, *Gambusia*/incipient toxic; *dark grey squares*, *Poecilia*/endemic toxic; and *dark grey circles*, *Gambusia*/endemic toxic.

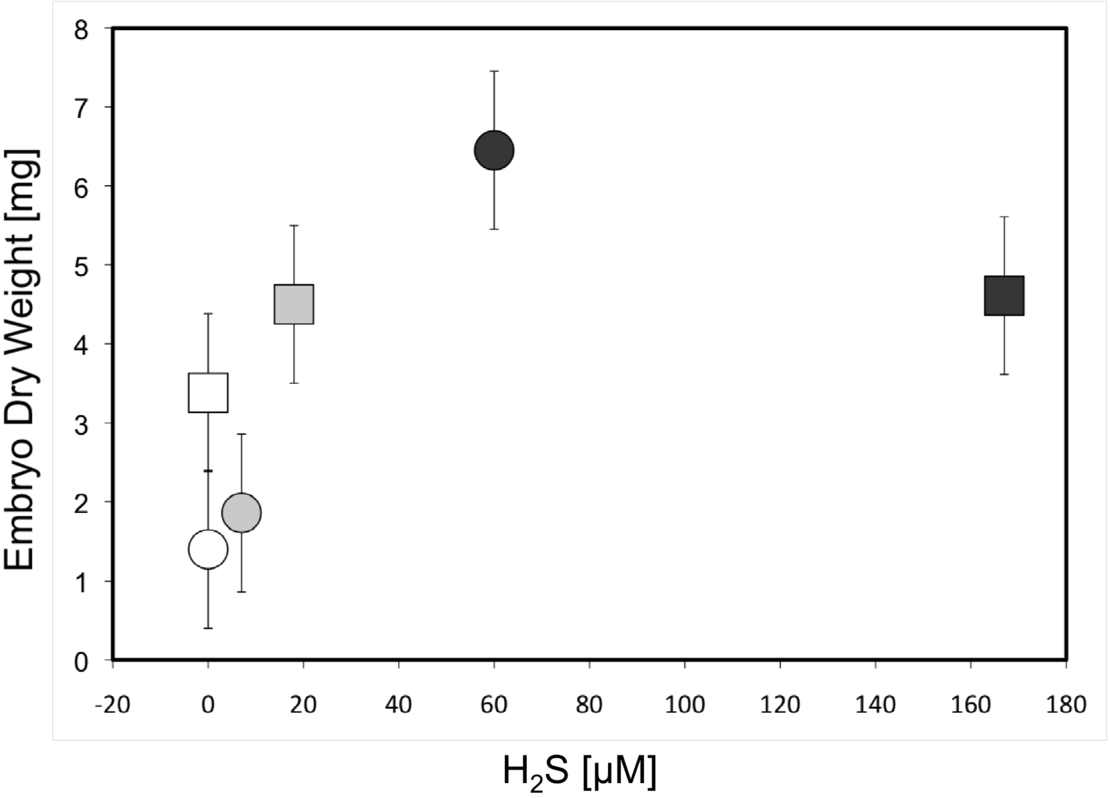

Embryo Dry Weight [mg]
0
1
2
3
4
5
6
7
8
-20
0
20
40
60
80
100
120
140
160
180
$H_2S$ [µM]

Figure 1

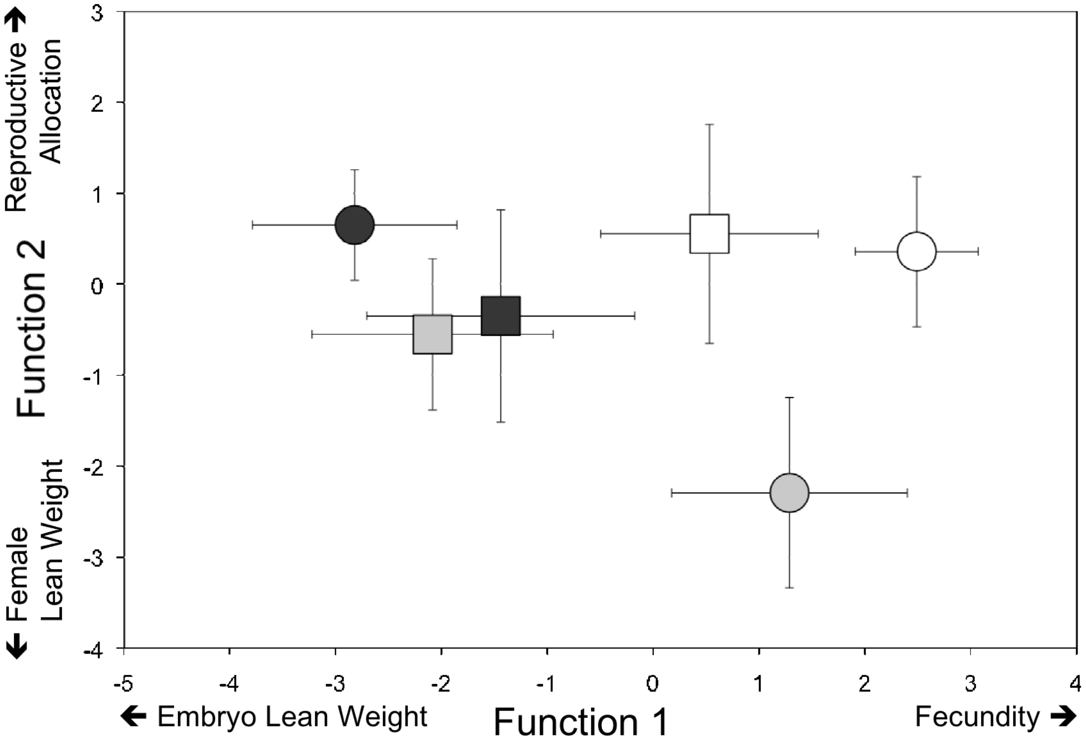
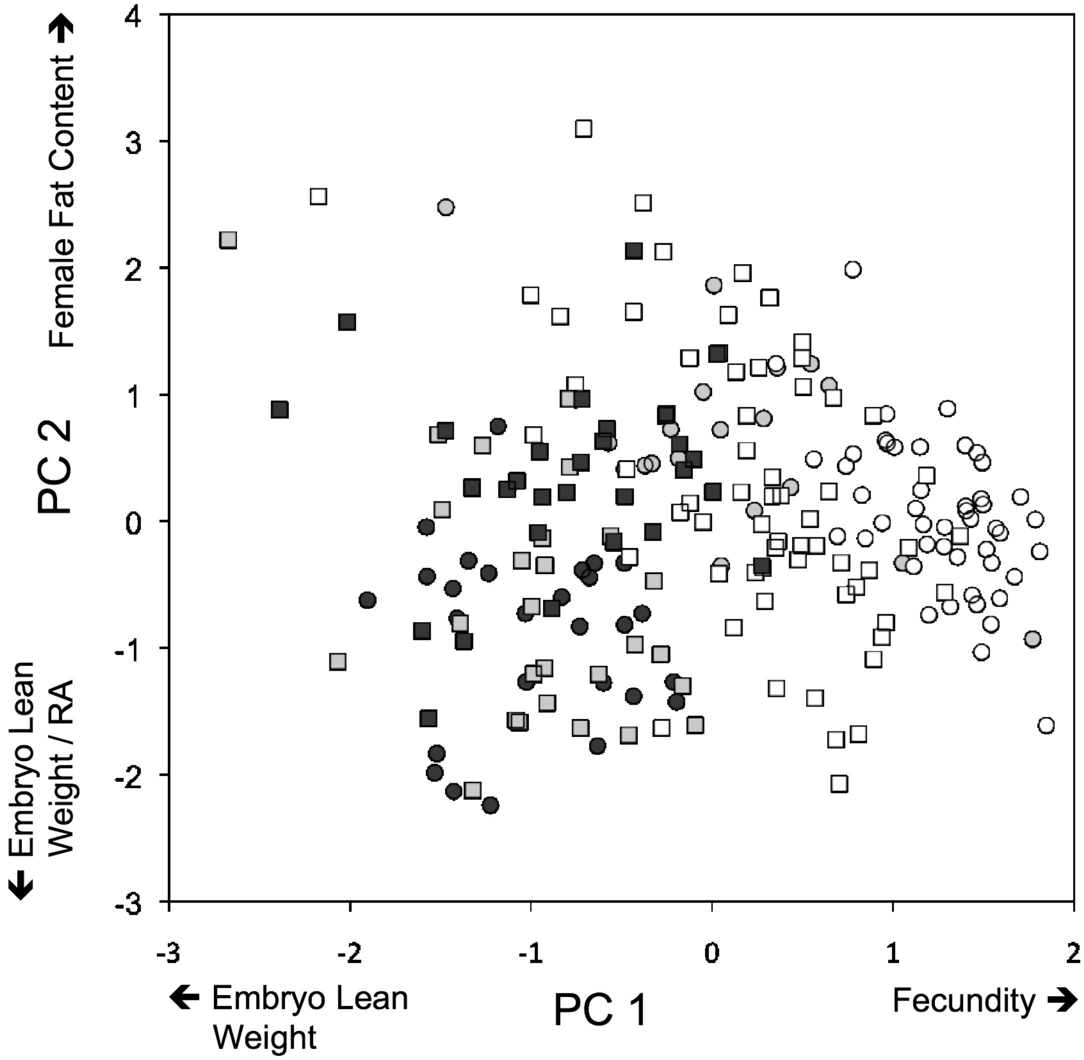

Reproductive ➔ Allocation
Function 2
🡐 Female Lean Weight
3
2
1
0
-1
-2
-3
-4
-5
-4
-3
-2
-1
0
1
2
3
4
🡐 Embryo Lean Weight
Function 1
Fecundity ➔

Figure 2



Female Fat Content →
PC 2
← Embryo Lean Weight / RA
4
3
2
1
0
-1
-2
-3
-3
-2
-1
0
1
2
← Embryo Lean Weight
PC 1
Fecundity →

Figure 3

# CHAPTER 4

# MATROTROPHY IN THE CAVE MOLLY: AN UNEXPECTED PROVISIONING STRATEGY IN AN EXTREME ENVIRONMENT

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Keywords: incipient matrotrophy, matrotrophy index, *Poecilia mexicana*, Poeciliidae, radio–tracer assay, viviparity

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# ABSTRACT

Maternal provisioning of animal embryos may be entirely through yolk deposited in the unfertilized egg (lecithotrophy) or may include post–fertilization nutrient transfer (matrotrophy) in varying degrees. Current theory suggests that the extent of post–fertilization provisioning is resource–dependent, with higher levels of matrotrophy being advantageous in more productive environments. In this study, we investigated post–fertilization embryo provisioning in a livebearing fish, *Poecilia mexicana*, from two different habitats (a toxic cave and a non-toxic surface habitat) that impose different energetic demands and therefore differ in resources available for reproduction. We predicted that fish in the benign habitat would be more matrotrophic than those from the toxic cave. We used two different techniques for this assay: (1) the matrotrophy–index analysis (MI) for field–collected fish and (2) both MI and radio–tracer assay for laboratory–reared females. According to the interpretation of the matrotrophy index, both populations are purely lecithotrophic, while the radio–tracer assay found females from both populations to actively transfer nutrients to developing embryos at approximately the same rate. Our results suggest that *P. mexicana*, which was traditionally classified as lecithotrophic, is capable of incipient matrotrophy, and that matrotrophy can contribute to embryo provisioning even in populations from resource–limited environments. Furthermore, the analysis of laboratory–reared animals provides evidence for a genetic component to the large offspring size in cave mollies, which had so far only been described from the field. Specifically, our results suggest matrotrophy occurs in species interpreted as lecithotrophic using the MI approach. Hence, to avoid misclassification, both techniques should ideally be

employed in concert, rather than individually. Finally, our results provide further insights into the possible evolutionary pathway from lecithotrophic oviparity to matrotrophic viviparity.

## INTRODUCTION

Nutrient transfer from mother to developing embryos (matrotrophy) occurs in numerous groups of animals and plants (Wourms et al. 1988; Marsh-Matthews in press) and substantial matrotrophy (see below) has independently evolved at least 24 times within vertebrates alone (Blackburn 1992, 2005a). Among matrotrophic forms, the relative importance of nutrient transfer in overall provisioning varies extensively (Blackburn 1992, 2005a), from contributing a minor amount of total nutrients and energy relative to yolk (incipient matrotrophy), to providing nearly all resources for embryonic development (substantial matrotrophy). Recent papers by Trexler and DeAngelis (2003) and Crespi and Semeniuk (2004) have suggested that the evolution of substantial matrotrophy has been a stepwise process, with a resource–dependent context for the evolution of matrotrophy from a lecithotrophic (yolk–only provisioning) ancestor (Trexler and DeAngelis 2003), which, once evolved, allowed for the evolution of extensive resource capture by embryos as an outcome of parent-offspring conflict (Crespi and Semeniuk 2004).

Livebearing fishes (family Poeciliidae) exhibit a wide range of post-fertilization nutrient transfer from incipient matrotrophy (Marsh–Matthews et al. in press) to substantial matrotrophy (Thibault and Schultz 1978; Wourms et al. 1988; Reznick et al. 2002), and many species were long assumed to be strictly lecithotrophic

(Reznick and Miles 1989; Marsh–Matthews in press). The group therefore provides unparalleled opportunities for comparative studies designed to elucidate factors associated with the evolution of matrotrophy of all levels (e.g., Reznick et al. 2002; Marsh–Matthews and Deaton 2006; Pires et al. 2007).

Trexler and DeAngelis (2003) modelled conditions favouring the evolution of incipient matrotrophy from a lecithotrophic ancestor. Based on the assumption that matrotrophic females produced more, but smaller, eggs than lecithotrophic females, and that post–fertilization provisioning to developing embryos resulted in the same offspring quality for the two provisioning modes, the model predicted that the matrotrophic strategy would be favoured only in environments with sufficient resources to sustain maternal provisioning throughout gestation. On the other hand, drastic fluctuations in resource-availability with frequent periods of very low resource availability is predicted to favour lecithotrophy (Trexler and DeAngelis 2003). In agreement with these predictions, there is evidence that the level of matrotrophy does vary with resource availability in livebearing fishes with substantial matrotrophy (Henrich 1988; Schrader and Travis 2005; Pires et al. 2007) as well as in those with incipient matrotrophy (Marsh–Matthew and Deaton 2006).

Among poeciliids with incipient matrotrophy (see Marsh–Matthews et al. in press), the Atlantic molly, *Poecilia mexicana*, provides a rare opportunity to examine ecological effects on maternal provisioning. *Poecilia mexicana* is widespread along the Atlantic versant of Mexico (Miller 2005), and in Tabasco, one population has invaded a sulfidic limestone cave, the Cueva del Azufre (Gordon and Rosen 1962). Inside the Cueva del Azufre several springs discharge water that is rich in hydrogen sulfide ($H_2S$), and while *P. mexicana* shares the surrounding benign surface habitats of the Río Oxolotán river system with a complex fish community, it is the only permanent piscine resident in the Cueva del Azufre (Tobler et al. 2006; Riesch et al. 2006, 2009a).

Even though caves are generally described as being resource–limited (Hüppop 2000; Poulson and Lavoie 2000), the Cueva del Azufre is different, because, as a sulfidic habitat, it is nutrient–rich due to chemoautotroph primary production (Langecker et al. 1996). However, since hydrogen sulfide is acutely toxic to metazoans and leads to extreme hypoxia in the water (Grieshaber and Völkel 1998; Tobler et al. 2006), *P. mexicana* from toxic habitats spend up to 84% of their time performing Aquatic Surface Respiration (ASR) to exploit the more oxygenated (and thus less sulfidic) top–most layer of the water column (Plath et al. 2007b; Tobler et al. 2009a). This leads to a drastic reduction in feeding time (Tobler et al. 2009a), transforming the Cueva del Azufre into a resource–limited environment for *P. mexicana*. Congruent with this interpretation, cave mollies have significantly less food in their intestinal tracts than surface mollies (Tobler et al. 2009a), have lower body condition (Plath et al. 2005; Tobler et al. 2006, 2008a; Tobler 2008), lower lean weights (Riesch et al. in press), and short-term survival in the cave is directly linked to access to high-energy food sources (Plath et al. 2007b). Since coping with $H_2S$ has been shown to be energetically costly (e.g., Sibly and Calow 1989; Ip et al. 2004), cave mollies probably funnel most of the available resources directly into immediate survival.

Tropical surface habitats show strong seasonal variations due to differences in temperatures and precipitation (i.e., rainy season vs. dry season), which also leads to high seasonal fluctuations in resource availability (Chapman et al. 1991; Chapman and Chapman 1993). Caves, on the other hand, are generally characterized by relatively constant temperatures year-round, and the only parameter known to vary in the Cueva del Azufre is the hydrogen sulfide discharge and any directly related water parameters (e.g., conductivity; Poulson & White 1969; Culver 2005; Tobler et al. 2006, 2008a, 2009b). However, this discharge varies from day to day and not so much from season to season (Hose and Pisarowicz 1999; Tobler et al. 2006, 2008a). Hence, resources are probably at a constant level year-round in the cave.

In the present study, we examined levels of incipient matrotrophy in both populations. For this, we utilized two approaches: (1) dissections of field–captured individuals and calculation of the matrotrophic index (e.g., Reznick et al. 2002), and (2) direct assay of nutrient transfer in laboratory populations (Marsh–Matthews et al. 2001, 2005). More specifically, we tested whether higher levels of incipient matrotrophy would be found in *P. mexicana* from (a) unpredictable resource–rich benign surface habitats or (b) a predictable resource-poor cave (Trexler and DeAngelis 2003).

## METHODS

### *Study system*

Fieldwork was conducted near Tapijulapa in Tabasco, Mexico, in June of 2007 and January of 2008. For the matrotrophy–index analysis *P. mexicana* were collected in the cave chambers V and X of the Cueva del Azufre (Gordon and Rosen 1962) and in the adjacent Río Oxolotán river system (sites: Río Amatan and Arroyo Bonita; see map in Tobler et al. 2008a), field preserved in 10% formaldehyde, and dissected in the laboratory of the University of Oklahoma (These data have been re-analyzed from a previous study: Riesch et al. in press).

The radio–tracer assay was conducted in July 2006, and *P. mexicana* came from 2nd to 3rd generation laboratory stocks of the two populations (Río Oxolotán and chamber XIII of the Cueva del Azufre; Gordon and Rosen 1962) that were established in September 2004 and refreshed with new wild–caught fish in January 2006. These stocks were maintained at the Aquatic Research Facility of the University of Oklahoma in 1000-L tanks in non–sulfidic water under natural-light conditions (i.e. sunlight) and *ad libitum* food.

### *Matrotrophy–index analysis (MI)*

This indirect method is the traditional approach to evaluating maternal provisioning by analyzing the relationship between embryonic dry weight and stage of development by means of regression analysis (e.g., Reznick et al. 2002; for embryonic stages please see Haynes 1995; Reznick et al. 2002). The matrotrophy index (MI) is then calculated as estimated embryo dry weight at parturition divided by the estimated dry weight of the unfertilized mature oocyte. In the case of lecithotrophy, the embryos should lose 30–40% of their dry weight during development (MI = 0.6–0.7) due to metabolism as observed in the eggs of oviparous species. On the other hand, if the provisional strategy is matrotrophy, the embryos either lose less weight (MI = 0.7–1.0) or they even gain weight during development (MI > 1.0; Scrimshaw 1945; Wourms et al. 1988; Reznick et al. 2002). We further performed Analyses of Covariance (ANCOVA) with log–transformed ‘embryo dry weight’ as the dependent variable, ‘population’ as the independent variable and ‘embryo stage’ as a covariate (Turcotte et al. 2008). A total of 29 mollies from the Cueva del Azufre and 63 from the Río Oxolotán river system were examined for the matrotrophy–index analysis.

### *Radio–tracer assay of matrotrophy*

This method is a direct measure for the occurrence of matrotrophy using $^3$H–leucine as a radio–tracer. A similar technique is commonly used to measure maternal transfer of nutrients to the offspring in other taxa (e.g., the lizard *Niveoscincus metallicus*, Swain and Jones 1997) and has successfully been used to determine maternal provisioning in other poeciliids (e.g., *Gambusia affinis* and *G. geiseri*, Marsh–Matthews et al. 2001, 2005). For a full protocol of the procedure refer to Marsh–Matthews et al. (2001, 2005). In short, pregnant females were injected with 8µl $^3$H–leucine (84 Ci/mmole) in the caudal peduncle and 2 h after the injection the females were euthanized using an overdose of MS–222 (Tricaine Methanesulfonate). In the ensuing dissection a sample of the liver was taken to assess uptake of the labeled source by the mother, and individual embryos were carefully dissected from the ovary to remove all maternal tissues, rinsed twice in distilled water and dried for 10 days at 40°C, weighed to the nearest 0.1 mg, and prepared for liquid scintillation assay as described by Marsh–Matthews et al. (2001, 2005) and Marsh-Matthews and Deaton (2006).

Nutrient transfer was quantified by measuring radioactivity present in individual embryos. Marsh-Matthew et al. (2005, for poeciliids) and Swain and Jones (1997, for a viviparous lizard) demonstrated that radiolabeled leucine is incorporated into the proteins of developing embryos within hours of injection of the mother. All embryo assays were done in the same scintillation run (with no correction necessary for counting efficiency), so radioactivity is expressed as counts per minute (cpm). Radioactivity was considered to be significantly greater than background level if the count rate of the sample minus its twofold sigma deviation (95% confidence interval) was greater than the count rate of the background sample plus its twofold sigma deviation (Marsh–Matthews et al. 2001). To assess uptake of the labeled leucine by the mother, radioactivity  was also measured in a liver sample of each female (Swain and Jones 1997; Marsh–Matthews et al. 2001, 2005).

A total of 20 mollies from the Cueva del Azufre and 19 from the Río Oxolotán were subjected to the direct assay for nutrient transfer. Fourteen mollies from the cave population had developing embryos. Of these, one was omitted from analyses because the liver was lost prior to scintillation assay. The remaining 13 individuals showed significant levels of radioactivity in both the liver and in embryos. For the surface population, only 11 females had developing embryos. Two of these females exhibited low uptake of the tritiated leucine as indicated by low radioactivity in the liver, and both were identified as outliers by examination of notched box plots (MedCalc 2008) with respect to liver radioactivity. [Of these two, one female did not exhibit significant radioactivity in embryos and another showed exceptionally low embryo radioactivity.]

These two females were omitted from further analyses, leaving a sample size of nine for the surface population.

Summary statistics of both maternal and brood characteristics were calculated for each brood subjected to scintillation assay. Total brood size included all developing embryos plus any unfertilized eggs and arrested or reabsorbing embryos. Calculations of mean embryo mass and mean embryo radioactivity (MER) included only developing embryos. Mean embryo radioactivity was based on log-transformed cpm for each developing embryo in the brood. Each brood was assigned a developmental stage based on the predominant stage present in the brood. All developing embryos dissected from injected females had distinguishable eye development, so the earliest stage of embryos in the radioassay was Stage 15 (Haynes 1995; Reznick et al. 2002).

Radioactivity detected in embryos is known to vary with the level of maternal uptake of the labeled nutrient as assayed by radioactivity in the maternal liver (Marsh-Matthews et al. 2005). To correct for variation due to differential uptake, we used residual embryo radioactivity calculated from least-squares regression analysis between embryo radioactivity and maternal liver radioactivity. In this study, mean embryo radioactivity was a linear function of liver uptake [LIV = log (liver cpm/liver mass)] by the mother (MER = 0.46LIV + 1.52; $R^2 = 0.52$, $p<0.001$). The two populations did not differ with respect to either slope or intercept of this relationship (ANCOVA) and were therefore pooled to calculate residuals of the least–squares regression between MER and LIV that were then used in further analyses of nutrient transfer. Residuals were normally distributed overall and for both populations separately (MedCalc 2008).

Nutrient transfer is known to sometimes vary with respect to several factors in poeciliids such as total brood size (Trexler 1997; Marsh–Matthews and Deaton 2006), embryo mass, and developmental stage (Marsh–Matthews et al. 2005). These variables were therefore included as covariates in an ANCOVA on the MER-residuals with population as a factor. To account for potential non-linear relationships, mean embryo size was log-transformed and total brood size was square-root-transformed. Finally, we ran an MI-analysis on the embryo-weight data from the radio-tracer assay, using an ANCOVA as described above for the original MI–analysis (Turcotte et al. 2008).

All tests were performed with SPSS 16.0 and MedCalc 2008. Variables were examined for normality (Kolmogorov–Smirnov test) and for the presence of outliers by examination of notched box plots (MedCalc 2008).

## RESULTS

### *Matrotrophy–index analysis*

Embryos lost weight in the course of gestation in both populations ($F_{1,88} = 23.10$, $p < 0.001$), however we did not find a significant interaction between populations and embryo stage ($F_{1,88} = 0.67$, $p = 0.72$), suggesting that no slope heterogeneity occurred (Fig. 1a). Indeed, the estimated MIs for both populations were virtually identical with a MI of 0.57 for the Río Oxolotán population and 0.56 for the Cueva del Azufre populations (Fig. 1a). However, the populations differed drastically from each other in embryo size, with embryos from the Cueva del Azufre ($N$ = 29) being roughly three times larger (estimated marginal mean of embryo weight corrected for stage: 8.79 mg) than embryos from the Río Oxolotán ($N$ = 63; estimated marginal mean: 3.40 mg) across stages (Fig. 1a). This was reflected by a significant effect of the factor 'population' ($F_{1,88} = 37.00$, $p < 0.001$).

### *Radio–tracer assay of matrotrophy*

With the exception of one female with atypically low uptake of the tritiated leucine, all surface and cave females exhibited significant nutrient transfer to developing embryos as assayed by injection of $^3$H-leucine into the mother. However, we could not detect a difference in the rate of nutrient transfer between populations ($F_{1,17} = 0.89$, $p = 0.36$; Fig. 2), nor did any of the covariates have a significant influence (total brood size: $F_{1,17} = 0.62$, $p = 0.44$; developmental stage: $F_{1,17} = 0.63$, $p = 0.44$; mean embryo mass: $F_{1,17} = 3.80$, $p = 0.07$).

Again, embryos lost weight in the course of gestation in both populations ($F_{1,18} = 14.18$, $p = 0.001$), but this time ANCOVA revealed a significant interaction of 'population by embryo stage' ($F_{1,18} = 6.24$, $p = 0.022$): while mean embryo dry weight declined steeply with developmental stage in cave mollies, it decreased only slightly in surface mollies (Fig. 1b). Cave mollies again produced larger offspring than surface mollies (estimated marginal mean of dry weight corrected for stage, cave: 5.260 mg, surface: 3.066 mg; Fig. 1b) and this was reflected by a significant effect of 'population' on embryo dry weights ($F_{1,18} = 22.00$, $p < 0.001$). However, relative to field–caught fish, embryo dry weight was lower across stages for lab–reared cave mollies (Figs. 1a and 1b).

## DISCUSSION

We analysed post–fertilization embryo provisioning in *P. mexicana* from two different habitats, a toxic cave and a benign surface habitat using two different techniques. According to the interpretation of the matrotrophy index, both populations are purely lecithotrophic, while the radio–tracer assay found females from both populations to actively transfer the amino acid to developing embryos. How exactly do we interpret these seemingly contradictory results we obtained from both methods? There are two non–mutually–exclusive explanations for this:

(1) The observed differences could in part be due to the fact that individuals for the radio–tracer assay came from the laboratory, while the fish for the MI–analysis were field–caught. However, it was logistically not possible to conduct the radio–tracer assay in the field. Therefore, even if the only reason for the observed differences were lab effects, it would still be evidence for the ability (whether realized in natural populations or not) of incipient matrotrophy in *P. mexicana*. Furthermore, additional *P. mexicana* populations from non-toxic surface habitats across México had similar MIs compared to the surface population investigated here (Riesch et al. in press), while *P. mexicana* from a recently discovered non-toxic cave (Tobler et al. 2008b) actually had an MI of 0.86 indicating low levels of maternal provisioning (i.e, incipient matrotrophy: Riesch et al. in press). Matrotrophy therefore appears to be a plastic trait in *P. mexicana* as suggested by Trexler (1997) for *P. latipinna*.

(2) One method could be more accurate than the other. For example, there are some limitations of the MI–analysis:

Firstly, it measures maternal provisioning only indirectly, by looking at mass of embryos at different stages of development rather than actual nutrient transfer. In species without superfetation (i.e., two or more broods are developing at the same time within the same female; Turner 1940; Scrimshaw 1944), this comparison must be made across different pregnant females (Marsh–Matthews in press). The underlying assumption is that embryos of lecithotrophic species should lose 30–40% (MI = 0.6–0.7) of their dry weight during development due to metabolism as observed in the eggs of oviparous species. However, this assumes a universal standard metabolism for oviparous teleosts, while at least in squamate reptiles these values vary considerably between species (e.g., Blackburn 1994; Stewart and Thompson 2000). It further assumes that metabolic rates of oviparous and viviparous embryos are the same, which may not be the case, because viviparous females can protect their offsprings' homeostasis through buffering from environmental fluctuations (Wourms et al. 1988; Blackburn 1999). Moreover, fish embryos of oviparous species developing in deposited eggs may actually have much longer or shorter developmental times (from fertilization to hatching) than the gestation period of the viviparous fish under investigation.

Secondly, the MI–analysis is not sensitive to differences in gestation period for the populations of viviparous fish under investigation. There are some indications in our populations that gestation period could be longer in cave mollies compared to their surface–dwelling relatives (Riesch, unpubl. data). If the populations (or species in other studies) under investigation actually differed in gestation period, then they would also differ in the rate of matrotrophy per time interval, even if they shared the same MI. Finally, the MI analysis does not provide a means to determine differential maternal provisioning at different stages of development (e.g., does provisioning take place at a constant rate, or only sporadically during specific stages of development?). This can only be inferred by looking at the individual data points, but not from the matrotrophy index.

The radio–tracer assay, on the other hand, is a direct means of detecting maternal nutrient transfer and it provides a clear-cut distinction between lecithotrophy and matrotrophy, because if radioactivity is detected in the embryo, nutrient transfer did take place. However, it is logistically very challenging to conduct the radio–tracer assay on field-caught fish. Therefore, it is most commonly employed on lab–reared fish, and one has to be extremely careful with interpreting its results in relation to the actual situation in field populations.

How then do we interpret our data with regards to wild cave and surface mollies? Our results suggest that the relative importance of matrotrophy differs between these environments. For cave mollies, embryo size was smaller in the lab–reared fish, but plasticity of offspring size is well–documented in poeciliids, especially with regards to variation in resource availability (e.g., Henrich 1988; Reznick and Yang 1993; Marsh–Matthews and Deaton 2006), so this was to be expected. Despite this, both surveys found that early–stage embryos were much larger in cave mollies than in the surface population, and embryo mass at these stages is primarily yolk (i.e., the lecithotrophic component is of greater importance at the onset of gestation in cave mollies). Even if reared in the laboratory under benign *ad–libitum*–food conditions (where we might expect cave mollies to maximize incipient matrotrophy according to Trexler and DeAngelis 2003; see also Marsh–Matthews and Deaton 2006) the initial maternal investment in yolk is still much greater than in surface mollies, which points to a genetic component to offspring size in cave mollies. In concordance with this, Riesch et al. (2009b) demonstrated a genetically inherited reduction in cave molly fecundity relative to surface mollies, but argued that this likely reflects selection for larger eggs, as would be predicted by a resource–dependent advantage of matrotrophy (Trexler and DeAngelis 2003). On the other hand, if nutrient transfer in the cave molly continued over a longer gestation time (see above), total matrotrophic investment per offspring might even be greater in the cave molly. A final answer to this question, however, needs to be postponed until quantitative common-garden data on interbrood intervals is collected.

The occurrence of, and perhaps dependence on, incipient matrotrophy in the cave molly may reflect phylogenetic inertia because a similar level of incipient matrotrophy occurs in the surface population from which the cave population was derived (Plath et al. 2007a; Tobler et al. 2008a). In fact, once evolved, matrotrophy may be like viviparity itself in that reversal to the presumed ancestral state (lecithotrophy in the case of nutrient transfer; oviparity in the case of livebearing) is highly unlikely, as suggested by Blackburn (2005b).

## *Conclusions*

Our study demonstrates that the designation of species as lecithotrophic based solely on the calculation of the matrotrophy index should be interpreted with extreme caution, and we propose that both techniques (MI and radio–tracer assay) should be employed in concert. Ideally, both techniques should be employed on both wild–caught and lab–reared animals of the same populations to investigate if and under which conditions the relative contribution of post–fertilization maternal–provisioning changes, as well as how the rate of matrotrophy contributes to embryo mass. As we outlined above, the matrotrophy–index analysis has the potential to 'predict' false negatives, i.e. small levels of matrotrophy may go undetected; however, the MI was the primary technique employed in the past to investigate evolutionary patterns of maternal transfer and placentation in poeciliids (e.g. Reznick et al. 2002, 2007; Turcotte et al. 2008). Recent analyses using the radio–tracer assay, however, have shown that a variety of traditionally–classified (based on MI analysis) lecithotrophic species are capable of incipient matrotrophy; in fact all species evaluated by Marsh-Matthews et al. (in press) exhibited incipient matrotrophy (*Gambusia affinis*, *G. clarkhubbsi*, *G. gaigei*, *G. geiseri*, *G. holbrooki*, *G. nobilis*, *Poecilia formosa*, *P. latipinna*, and *P. mexicana*). Furthermore, recent studies have stressed that provisioning strategies represent a continuum rather than a true dichotomy, with pure lecithotrophy and pure matrotrophy at the endpoints and different combinations of relative degrees of prefertilization– vs. postfertilization–provisioning in between (e.g., Pires et al. in press). More specifically, pure matrotrophy would require embryo development without production of yolk. However, in the family Poeciliidae fertilization always involves yolked ova (Kunz 2004) so a pure matrotrophic strategy is not possible. Furthermore, due to recent discoveries of incipient matrotrophy among supposed ‘lecithotrophs’ (this study; Trexler 1985, 1997; DeMarais and Oldis 2005; Marsh–Matthews et al. in press) it now also seems unlikely that any poeciliids (with the possible exception of basal *Tomeurus gracilis*, which is facultatively oviparous; Constantz 1989) are purely lecithotrophic. Hence, current theories on the evolution of matrotrophy and lecithotrophy in poeciliid fishes need to be revised (e.g., Reznick et al. 2002, 2007; Turcotte et al. 2008), as poeciliids are probably characterized by dual provisioning (for dual provisioning in squamate reptiles see also Stewart and Thompson 2000; Thompson et al. 2000). This interpretation would lend further support to Blackburn’s (1992, 1999, 2005b) hypothesis of coevolution of viviparity and matrotrophy, rather than the traditional view of having matrotrophic viviparity evolve from lecithotrophic viviparity.

Finally, studies of resource effects on incipient matrotrophy, and its relative role in offspring provisioning can establish necessary conditions for, and constraints on, the evolution of matrotrophy, and thus may begin to explain the multiple, independent origins of this widespread provisioning strategy.

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# FIGURE LEGENDS

**Figure 1.** Matrotrophy–index analysis: Mean embryo dry weight [mg] versus stage of development of *Poecilia mexicana* from (**a**) wild–caught females and (**b**) lab–reared specimen. Stages are determined on a progressive scale with the earliest (5) being the neurula stage and the oldest (50) being equivalent to ready–to–be–born embryos (after Haynes 1995; Reznick et al. 2002). Fish come from the Río Oxolotán river system (*white circle*) and the Cueva del Azufre (*black circle*).

**Figure 2.** Box plots for the cpm residuals (embryo radioactivity corrected by a least-squares regression of embryo radioactivity on liver activity) in both populations of *Poecilia mexicana* as derived from the radio–tracer assay of maternal provisioning.

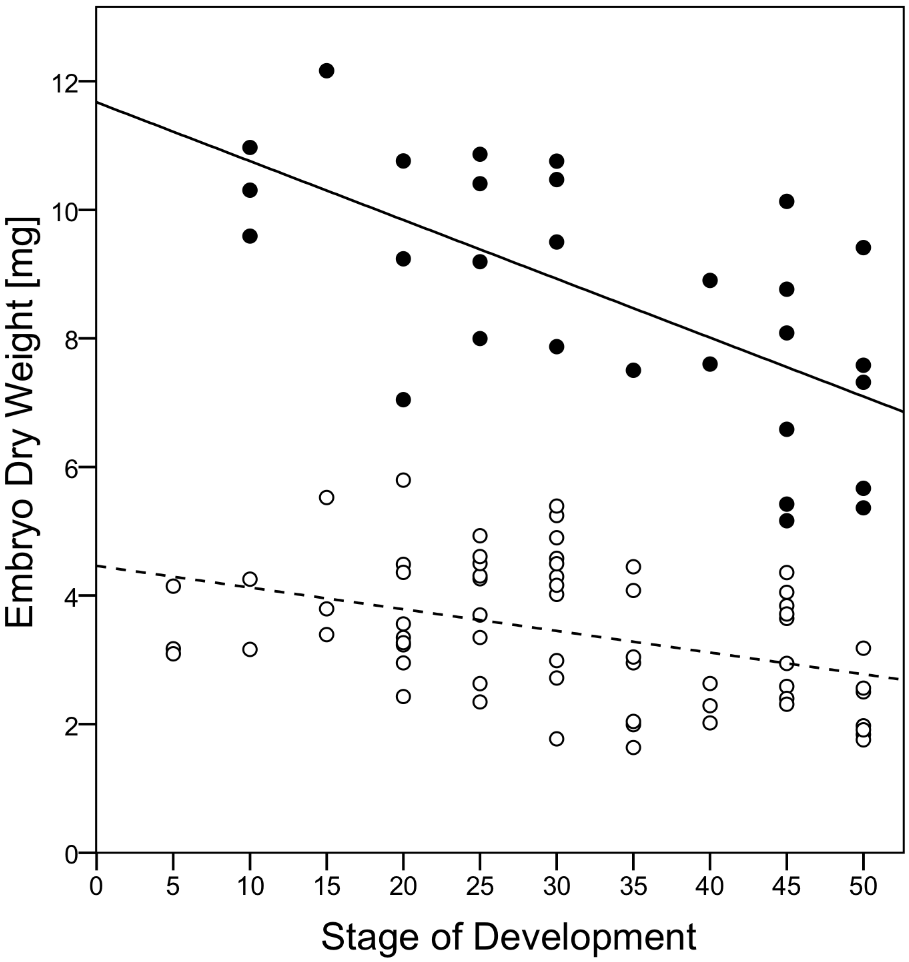
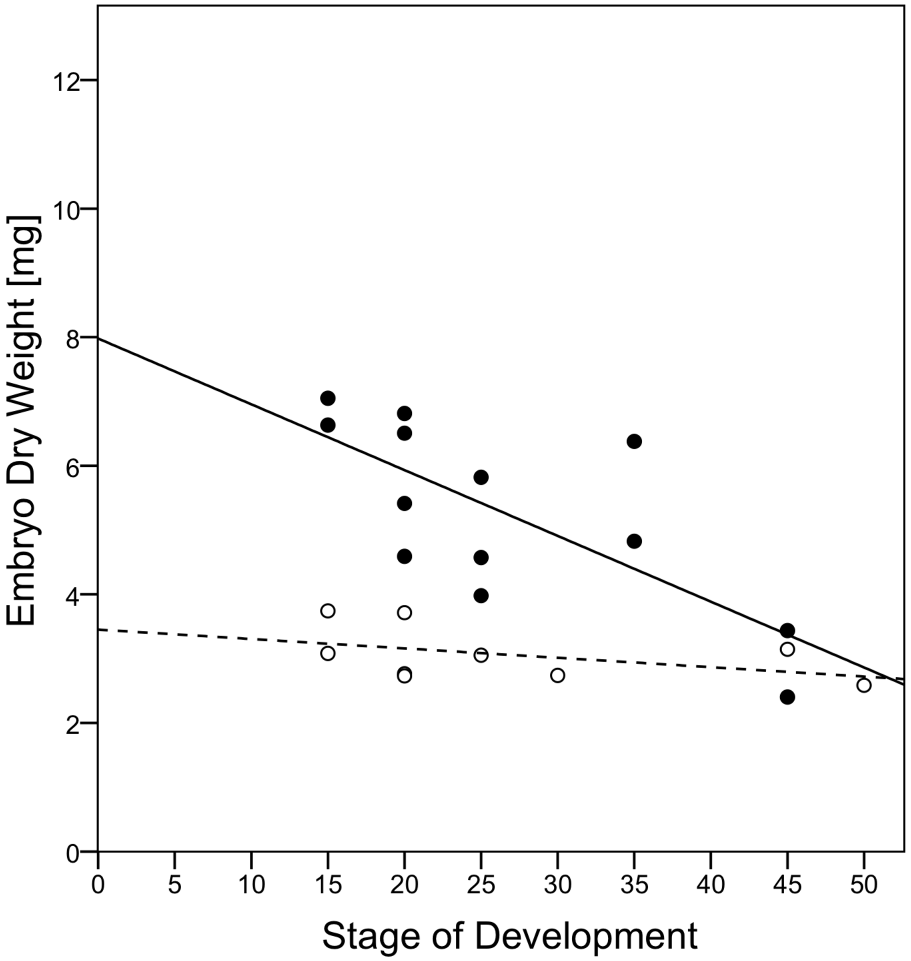
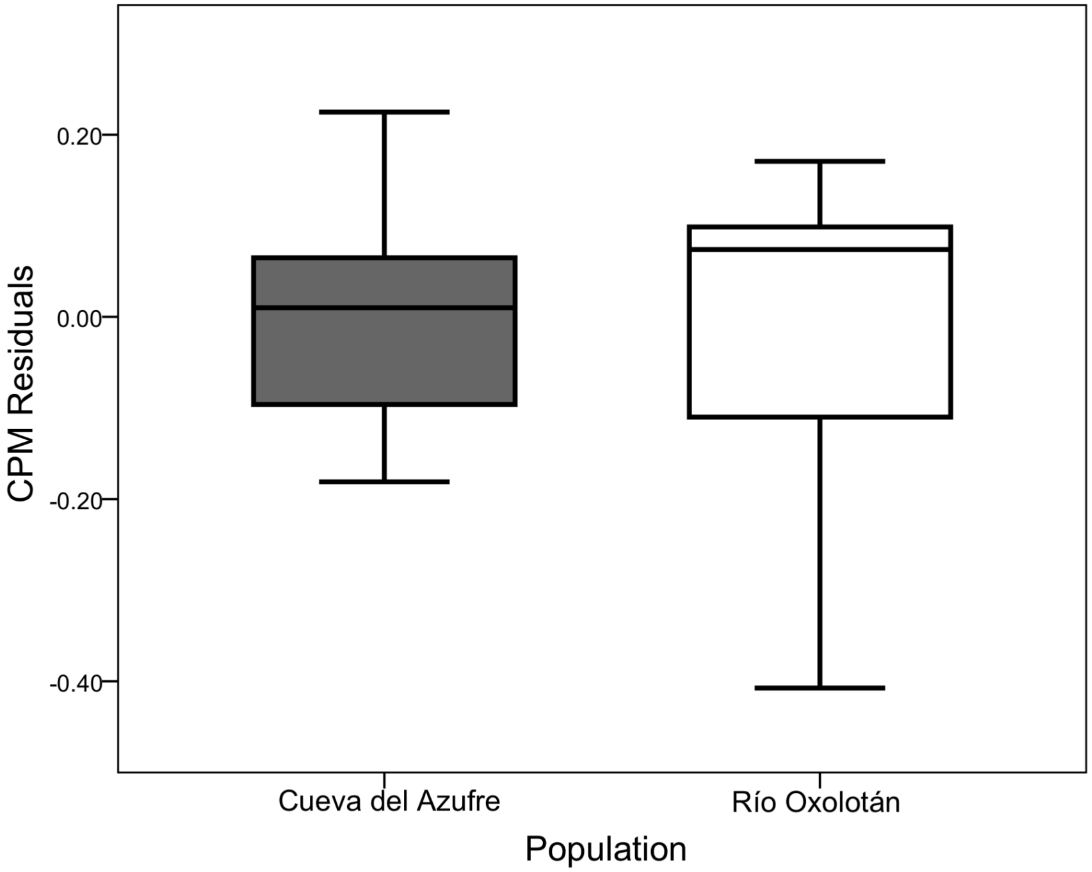

Embryo Dry Weight [mg]
0
2
4
6
8
10
12
0
5
10
15
20
25
30
35
40
45
50
Stage of Development

Figure 1a



12
10
8
6
4
2
0
Embryo Dry Weight [mg]
0
5
10
15
20
25
30
35
40
45
50
Stage of Development

Figure 1b


0.20
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-0.20
-0.40
CPM Residuals
Cueva del Azufre
Río Oxolotán
Population

Figure 2

# CHAPTER 5

# FEMALE SPERM-LIMITATION IN NATURAL POPULATIONS OF A SEXUAL/ASEXUAL MATING-COMPLEX (*POECILIA LATIPINNA*, *POECILIA FORMOSA*)

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Keywords: Amazon molly, male mate choice, mate preference, sperm transfer, gynogenesis, Poeciliidae

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## ABSTRACT

In sperm-dependent sexual/asexual mating systems, male mate choice is critical for understanding mechanisms behind apparent stability observed in natural populations. The gynogenetic Amazon molly (*Poecilia formosa*) requires sperm from sexual males (e.g., *Poecilia latipinna*) to trigger embryogenesis, but inheritance is strictly maternal. Consequently, males should try to avoid or reduce the cost of mating with asexuals. We investigated male mate choice by documenting the presence of sperm in natural populations and found that a higher proportion of sexual females had sperm than asexuals. Also, among those females that had sperm, sexuals had more sperm than asexuals. Our results hint at a role for male mate choice as a stabilizing factor in such systems.

## INTRODUCTION

Understanding the evolution and maintenance of sexual reproduction remains a major challenge in evolutionary biology (West *et al.* 1999). Asexual, all-female species produce twice as many daughters and thus, should quickly out-compete sexuals in the short run (Maynard Smith 1978; Bell 1982; Barton & Charlesworth 1998). Some asexual species, however, need to mate with males of heterospecific sexual species, the sperm of which triggers embryogenesis; this mechanism is called gynogenesis (Beukeboom & Vrijenhoek 1998; Schlupp 2005). In our study system, a gynogenetic fish (*Poecilia formosa*) of hybrid origin relies on sperm from host males of the two parental species (*P. latipinna* or *P. mexicana*). Here, local extinction of the sexual species would also bring about the local extinction of the asexuals. How can such a sexual/asexual mating-complex be stable?

Two main types of regulatory mechanisms have been proposed. Ecological disadvantages for asexuals could explain the stability of mixed sexual/asexual systems. One example would be the "Red Queen hypothesis", which states that recombination produces genetically diverse offspring that are harder to target by pathogens than the clonal asexuals (Van Valen 1973). However, currently no evidence supports this explanation in *P. latipinna* and *P. formosa* as they do not differ significantly in parasite loads (Tobler & Schlupp 2005). Alternatively to ecological disadvantages, the "behavioural regulation hypothesis" assumes that male mate choice regulates the system (reviewed in Schlupp 2005). In laboratory experiments, both *P. latipinna* and *P. mexicana* males have been shown to prefer mating with conspecifics (reviewed in Schlupp 2005), and *P. latipinna* males to prefer larger over smaller females regardless of species (Gumm & Gabor 2005). Obviously, male rejection of heterospecific females would be adaptive and mating preferences for conspecifics are both predicted by theory and described in other sexual/asexual mating complexes (McKay 1971; Løyning & Kirkendall 1996; Engeler & Reyer 2001). Previous laboratory studies were able to research male choice on the behavioural level, including differential sperm production (Aspbury & Gabor 2004) and sperm transfer (Schlupp & Plath 2005), but so far studies investigating male choice under natural conditions have been missing.

We conducted a field study testing for male mate choice in mixed populations of *P. latipinna* and *P. formosa*. We compared the amount of sperm found in the genital tract of sexual and asexual females as a proxy for male mate choice. Based on a recent metapopulation model male mate choice and sperm-limitation of asexuals are two factors predicted to drive such species complexes towards stability (Kokko *et al.* 2008). Hence, this is the first study that links behavioural patterns observed in the lab with fitness-relevant sperm transfer in the wild.

## METHODS

### *Study populations*

*Poecilia latipinna* and syntopically occurring *P. formosa* were collected at five sites in Central Texas (San Marcos River drainage, Caldwell County, sites 1-3) or Southern Texas (Rio Grande Valley, Hidalgo and Cameron County, sites 4-5). Sites 1 and 2 lie in the residential area of Martindale (29°51.48'N 097°51.85'W). Site 1 consists of a large pond, which drains across a street into a creek and ultimately into the San Marcos River. Site 2 is only about a hundred meters upstream from Site 1, and is a small partly spring-fed backwater of the San Marcos River. Site 3 is located at the San Marcos River a few kilometres downstream from the Aquarena Springs springhead in San Marcos (29°51.43'N 097°53.80'W). Site 4 in southern Texas is located in the City of Brownsville, Cameron County (25°53.98'N 097°28.77'W). It is an oxbow lake approximately 2 km north of the Rio Grande, situated directly underneath an Express-Highway overpass. Finally, site 5 is part of the irrigation system of the Progreso-area of Hidalgo County (26°07.20'N 097°57.67'W) and belongs to the 'Llano Grande lake Arroyo Colorado'-watershed. Originally, we had planned to include a third southern Texas site at the State fish hatchery in Olmito, Cameron County. However, although it had been a successful field-site for previous years (e.g., Heubel 2004), we did not find any Sailfin or Amazon mollies there in 2006.

Both species were kept at densities of 30-35 fish in aerated Styrofoam coolers in approximately 5 l of water, and transported to a nearby field lab for sperm analyses. We assessed the relative frequencies of mature males and females (sexual and asexual) during each field trip by conducting six standardized hauls using a standard seine (4 m long, 4 mm mesh width) and consistently covering the same area (Table 1).

### *Field experiment*

At the beginning of our study (all field sites in March and two sites in June) we kept females and males at an approximate ratio of 10:1 together in the same cooler for about 60-240 minutes. This was an unintended experiment, but it gave males and females an opportunity to mate after being captured (series 1). To avoid these matings and focus only on the matings that happened in nature, all subsequent sperm measurements were conducted on females that were separated from males upon capture (series 2).

### *Semen extraction and sperm counts*

Semen extraction and sperm counts followed standard protocols (e.g., Evans *et al.* 2003; Schlupp & Plath 2005). All sperm extractions were carried out in a randomized order with respect to females of each species. Females were anaesthetized using MS222, then rinsed with fresh water, and placed belly-up on a wet paper towel, so that head and gills were embedded in wet paper. Sperm was transferred into Eppendorf tubes containing 50 µl frog Ringer solution. Using a stereomicroscope, sperm was retrieved from the females by introducing a small flexible plastic micropipette (containing an aliquot of the 50 µl Ringer solution) into their genital opening (Luyten & Liley 1991; Schlupp & Plath 2005). To flush out the female genital tract, the solution was gently injected and retrieved three times (Evans *et al.* 2003). The solution was then transferred into the original Eppendorf tube, combined to 50 µl, and gently shaken to break up the spermatozeugmata. Male poeciliids transfer their spermatozoa in tightly packed bundles, so-called spermatozeugmata, which under natural conditions do not break apart until after being transferred into the female genital tract (Constantz 1989).

Two drops of the sperm-containing solution were transferred to a *Neubauer improved* counting chamber and covered with a slide. We decided *a priori* that spermatozoa touching the upper and right line of a field were counted for the sample, while those touching the lower and left line were omitted. Spermatozoa were counted in six randomly selected fields and the mean was calculated. From the resulting data for the known volume of the counting chamber, the total number of sperm was calculated (Schlupp & Plath 2005).

### *Statistical analyses*

All tests were calculated separately for series-1 sampling times and series-2 sampling times using SPSS 11 (SPSS Inc (2002). In series 1, the frequency of females with sperm was compared between both types of females using logistic regression. In series 2, ‘time of sampling’ (as proxy for time of year) was included as another factor. To test for differences between species in amount of sperm flushed out, females without sperm in their genital tract were excluded from the analysis. Because data were not normally distributed even after attempting transformation, non-parametric *U*-tests were employed. Female standard length was compared between species using *t*-tests.

### *Validation of the technique*

To validate that the applied technique is equally successful in retrieving sperm from both types of females, a laboratory-experiment was conducted. Eight females of each species were artificially inseminated with known amounts of sperm cells (range: 134,500,000 to 558,000,000), and sperm was retrieved after either 0, 30, 60, or 120 minutes. In a GLM, 'log(fraction of sperm retrieved)' was the dependent variable and 'time' and 'species' were included as categorical factors. Species identity had no significant effect (mean square = 1.368E-5, $F_{1,13} = 0.064$, $p = 0.804$), but time of retrieval had (mean square = 1.680E-2, $F_{1,13} = 79.020$, $p < 0.001$): the relative amount of sperm retrieved decreased over time ($R^2 = 0.847$).

Finally, 120 minutes after initial insemination, only trace amounts of sperm were detected. However, it is important to note that we had no intention of determining how long sperm could be flushed out of the female genital tract. We know from previous experience that we can flush out sperm several days after copulations (in guppies sperm can be flushed out of the genital tract up to 7 days after copulation: Clark & Aronson 1951; Liley 1961). Hence, the fact that only trace amounts of sperm could be detected after 120 min in our experiment does not mean that sperm we find in field-caught females cannot be older than 2 hours. For our experiment, we conducted artificial inseminations, which means that we (1) most likely transferred less sperm than during a natural insemination, and (2) also widened the genital pore considerably during the insemination process, so that a large proportion of the transferred sperm most likely leaked out of the female immediately following insemination.

## RESULTS

### *Mate choice and sperm transfer with males in the coolers*

After being transferred from field sites to the laboratory together with males, a significantly higher proportion of sexual females had sperm in their genital tract (Table 2; Fig. 1). Among those females that had sperm, *P. latipinna* females (total sperm, Median: 783,333.5, IQR: 175,000, $N = 30$) had significantly more sperm than *P. formosa* (Median: 66,667, IQR: 141,667.3, $N = 28$, $U_{56} = 208.500$, $p < 0.001$), although *P. formosa* were on average larger (*P. latipinna*: 41.75±9.71mm (Mean±S.D.), $N = 51$; *P. formosa*: 45.94±8.97mm, $N = 72$; $t_{121} = -2.473$, $p = 0.015$).

### *Mate choice and sperm transfer under natural conditions*

A significantly higher proportion of *P. latipinna* females had sperm in their genital tract than *P. formosa* when no males were in the coolers (Table 2; Fig. 1). Time of year ('time of sampling') also had a significant influence on the proportion of females with sperm (Table 2; Fig. 2): during June 10.7±6.0% (Mean±S.E.) of *P. formosa* had sperm, this number increased to 20.7±5.4% in July, but then dropped to 2.3±2.3% and 3.6±2.5% in August and September, respectively. In June 66.7±11.4% of *P. latipinna* had sperm, this number decreased to 26.3±7.2% in July, 10.5±7.2% in August, and 8.9±4.3% in September (Fig. 2).

Among those females that had sperm in their genital tract, no significant difference was found between species in the total number of sperm retrieved (*P. latipinna*: Median: 216,666.5, IQR: 800,000, $N$ = 36; *P. formosa*: Median: 83,333, IQR: 107,666.5, $N$ = 21, $U_{55}$= 179.500, $p$ = 0.101). Again, *P. formosa* were larger (*P. latipinna*: 39.78±7.40mm, $N$ = 120; *P. formosa*: 42.69±7.79mm, $N$ = 185, $t_{303}$ = 3.202, $p$ = 0.002).

### *Species and sex ratio*

Throughout our study, *P. formosa* was always the more common species. In addition, female *P. latipinna* outnumbered males (Table 1; Fig. 2). However, an interesting shift occurred in August/September because males became disproportionally rare, while the ratio of female *P. latipinna* to *P. formosa* remained largely unchanged (Table 1; Fig. 2).

## DISCUSSION

Our study resulted in two main findings. First, a higher proportion of *P. latipinna* than *P. formosa* had sperm in their genital tracts. This was true for series 1, in which matings were possible inside coolers, but – most importantly – also for series 2, where sperm transfer under natural conditions could be determined. Second, sexual *P. latipinna* had more sperm under all conditions (although not always statistically significant), even when males became exceedingly rare towards the end of the breeding season. These results are consistent with the predictions of a recent metapopulation model on the stability of this sexual/asexual mating complex (Kokko *et al.* 2008). Furthermore, our results are in agreement with behavioural studies in several other asexual/sexual mating systems, where males prefer sexual females over asexual females in the laboratory (reviewed in Schlupp 2005). Also, by interpreting pregnancy rates, asexual *Poeciliopsis monacha-lucida* were found to lack sperm more often than sexual *P. lucida* (McKay 1971). However, very few studies have directly looked at the role of sperm (but see Aspbury & Gabor 2004, Schlupp & Plath 2005).

We also found several other interesting patterns: *P. formosa* was more common than *P. latipinna*, and Amazon molly females were larger than their sexual counterparts during all field trips. Since under laboratory conditions *P. latipinna* males prefer larger females to smaller ones (Gumm & Gabor 2005), wild males apparently prefer to mate with *P. latipinna* females despite their smaller body size. These patterns are consistent with male mate choice operating in this system under natural conditions and allow male mate choice to contribute to the stabile coexistence of sexual and asexual forms by reducing fitness of *P. formosa*. However, we cannot rule out that other factors, e.g., female competition for mating, play a role here (reviewed in Schlupp 2005).

Hubbs (1964) found that wild-caught *P. formosa* and *P. latipinna* females produce approximately the same number of eggs, but a higher proportion of these actually develop into embryos in *P. latipinna*. Theoretically, this difference in fecundity (i.e., number of developing embryos) could be explained by two scenarios: beside sperm-limitation, lower fecundity in *P. formosa* could also be driven by other mechanisms, like developmental instability due to accumulation of deleterious mutations in clonal, non-recombining asexuals (*sensu* Muller 1964). However, fecundity (i.e., number of offspring per female) of lab-reared *P. formosa* does not differ from that of equal-sized *P. latipinna* (Schlupp *et al.* in press); which further suggests that sperm-limitation in nature due to male mate choice plays a substantial role in stability of this sexual/asexual mating complex.

The proportion of females with sperm fluctuated over the summer, and decreased towards the end of the season in both species. Interestingly, the ratio of female *P. latipinna* to *P. formosa* did not change much in our study. Males, however, became proportionally more rare during the course of the breeding season. It has been suggested that male choice is negatively frequency-dependent in this and other sexual/asexual mating systems (reviewed in Schlupp 2005), with males preferring the rare species and discriminating against asexuals only when conspecific females are rare. This question has been addressed in another poeciliid, *Poeciliopsis*, where several studies demonstrated that mating success of asexuals was inversely correlated with density (e.g., Moore & McKay 1971; Stenseth *et al.* 1985; but see Keegan-Rogers & Schultz 1988). Our data, however, do not support negative frequency-dependency of male mate choice, since *P. formosa* was always the more common species. Rather our data mirror Løyning & Kirkendall's (1996) findings in the bark beetle *Ips acuminatus*, where males generally discriminate less when the ratio of males to females decreases.

In summary, compared to their sexual counterparts, only a small proportion of *P. formosa* successfully acquires sperm in syntopic populations. Furthermore, they most likely also receive less sperm than *P. latipinna*. Regardless of how much sperm is really needed to fertilize a whole clutch, this strongly suggests that wild *P. formosa* are sperm-limited and underscores the role of male mate choice as a stabilizing factor in this system (see also Kokko *et al.* 2008). However, more research is needed to investigate how male mate choice influences life-history characteristics in this and other sexual/asexual mating complexes.

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**Table 1.** Total frequencies of *Poecilia formosa*, and male and female *P. latipinna* for each month in all populations combined.

| Month | *Poecilia latipinna* (males) | *Poecilia latipinna* (females) | *Poecilia formosa* |
|---|---|---|---|
| June | 14 | 17 | 103 |
| July | 22 | 53 | 97 |
| August | 5 | 42 | 115 |
| September | 34 | 117 | 416 |

**Table 2.** Logistic regression on the frequency of females with sperm in their genital tract.

| | -2 Log likelihood | B | S.E. | *Wald* | *df* | *p* |
|---|---|---|---|---|---|---|
| *a) Females with males in cooler (Chi-Square$_1$=9.033; p=0.003)* | | | | | | |
| Species | 79.773 | -1.804 | 0.679 | 7.053 | 1 | **0.008** |
| *b) Females without males in cooler (Chi-Square$_1$=15.696; p<0.001)* | | | | | | |
| Species | 219.747 | -1.148 | 0.349 | 10.808 | 1 | **0.001** |
| Time of Sampling | 219.747 | | | 23.453 | 3 | **<0.001** |

# FIGURE LEGENDS

**Figure 1.** Pie charts depicting the percentage of females with sperm in their genital tract (white) to females without (black). Top half: females kept together with males prior to sperm analysis; Lower half: females kept separate from males prior to sperm analysis. Left column: *Poecilia latipinna*; right column: *P. formosa*.

**Figure 2.** Combined line and bar graph: (1) lines representing the proportion of *P. latipinna* and *P. formosa* females with sperm in their genital tract on four separate field trips (all females were kept isolated from males prior to sperm analysis), and (2) bars representing the total number of females per male in all populations combined on the day of sampling.

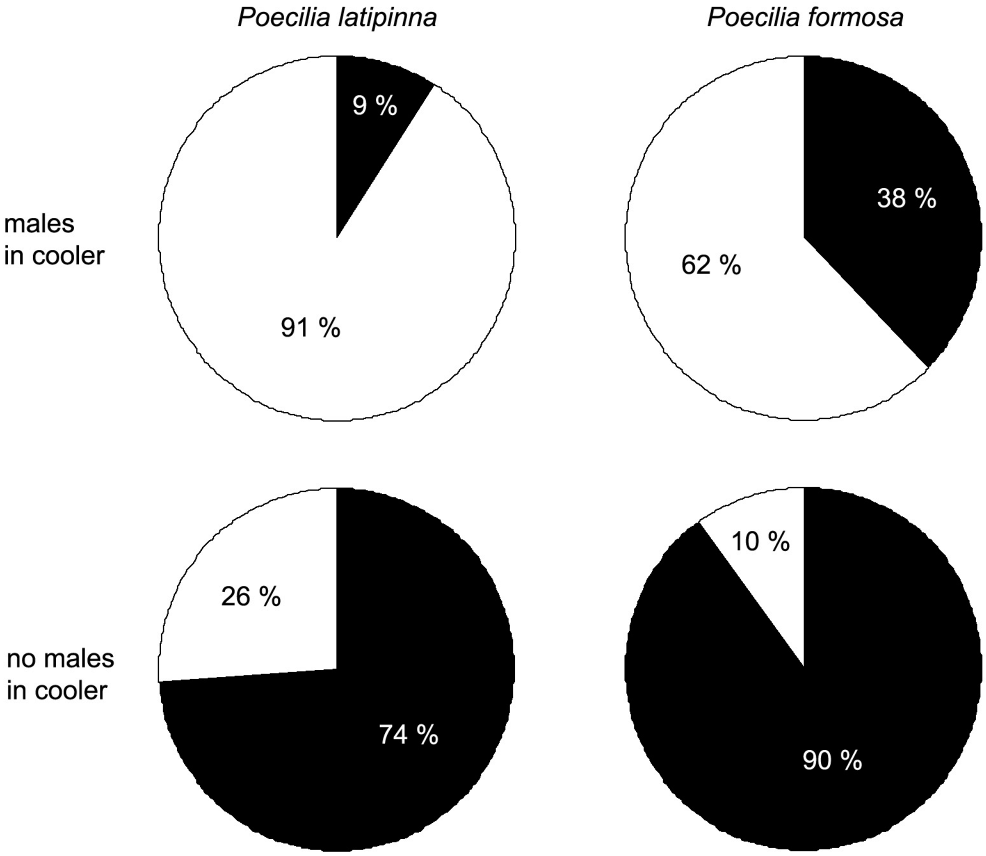
Poecilia latipinna
Poecilia formosa
males
in cooler
9 %
91 %
38 %
62 %
no males
in cooler
26 %
74 %
10 %
90 %

Figure 1

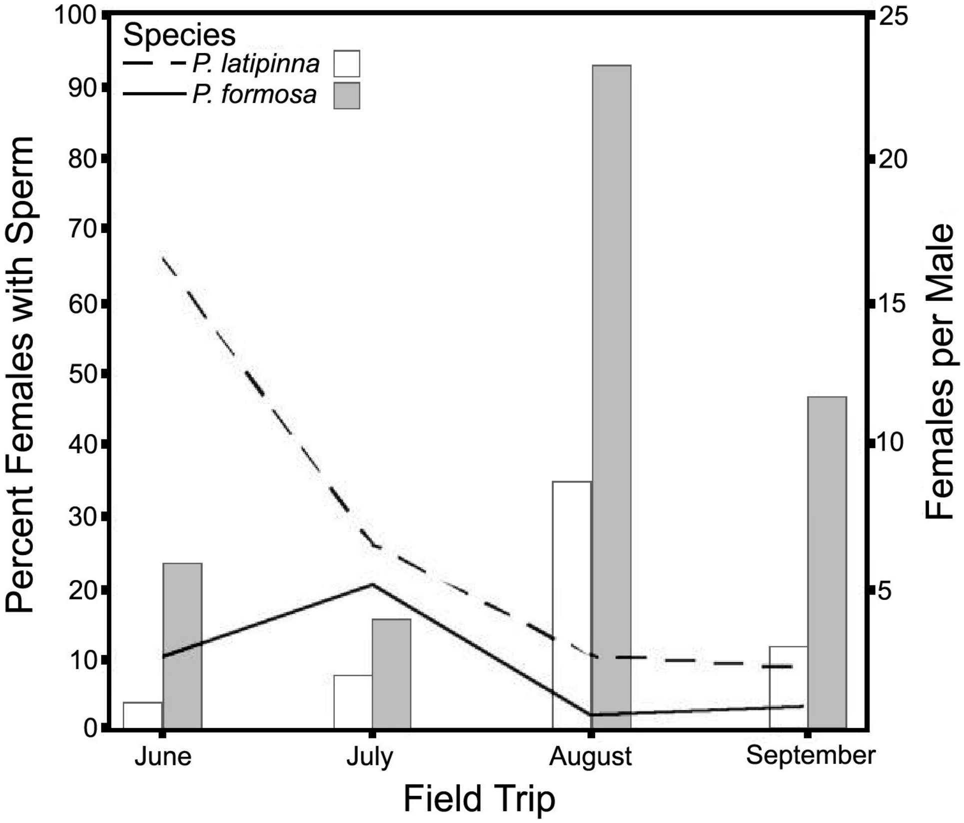
Species
P. latipinna
P. formosa
Percent Females with Sperm
Females per Male
Field Trip
June
July
August
September
0
10
20
30
40
50
60
70
80
90
100
5
10
15
20
25

Figure 2

# APPENDIX A

# SUPPLEMENTARY MATERIAL TO CHAPTER 2

Scheduled to be published as online Appendix A and B in 2010, *Ecology* **91(5)**

This version differs from the published version:

*(a) Text of online Appendix A was incorporated into the main text of chapter 2*

*(b) Tables and figures of online Appendices A and B were combined in Appendix A*

**Table A1.**General linear model (GLM) on female condition in four Mexican drainages and habitat types. SL = standard length. All dependent variables and covariates were log–transformed (or arcsine–transformed if percentages) to accommodate for a potential non–linear relationship between the variables. Only significant results are shown.

| Factor | Dependent Variable | *df* | Mean Squares | *F* | *p* |
|---|---|---|---|---|---|
| SL [mm] | Female Lean Weight [mg] | 1 | 4.613 | 1902.660 | <0.001 |
| Drainage | Female Fat Content [%] | 2 | 0.142 | 17.931 | <0.001 |
| | Female Lean Weight [mg] | 2 | 0.087 | 35.813 | <0.001 |
| Light Condition | Female Lean Weight [mg] | 1 | 0.009 | 3.879 | 0.050 |
| Toxicity | Female Fat Content [%] | 1 | 0.351 | 44.174 | <0.001 |
| Light Condition*SL | Female Lean Weight [mg] | 1 | 0.014 | 5.574 | 0.019 |
| Light Condition*Toxicity | Female Fat Content [%] | 1 | 0.080 | 10.081 | 0.002 |
| | Female Lean Weight [mg] | 1 | 0.038 | 15.804 | <0.001 |

**Table A2.**General linear model (GLM) on female reproduction in four Mexican drainages and habitat types. SL = standard length. All dependent variables and covariates were log–transformed (or arcsine–transformed if percentages) to accommodate for a potential non–linear relationship between the variables. Only significant results are shown.

| Factor | Dependent Variable | *df* | Mean Squares | *F* | *p* |
|---|---|---|---|---|---|
| SL [mm] | Fecundity | 1 | 1.838 | 50.758 | <0.001 |
| | Embryo Lean Weight [mg] | 1 | 0.133 | 7.946 | 0.005 |
| Embryonic Stage | Reproductive Allocation [%] | 1 | 0.033 | 6.652 | 0.011 |
| Fat Content [%] | Fecundity | 1 | 0.328 | 9.056 | 0.003 |
| | Reproductive Allocation [%] | 1 | 0.157 | 31.631 | <0.001 |
| Drainage | Reproductive Allocation [%] | 2 | 0.018 | 3.654 | 0.028 |
| Light Condition | Fecundity | 1 | 4.126 | 113.923 | <0.001 |
| | Embryo Lean Weight [mg] | 1 | 1.997 | 119.500 | <0.001 |
| | Embryo Fat Content [%] | 1 | 0.158 | 63.888 | <0.001 |
| | Reproductive Allocation [%] | 1 | 0.020 | 4.013 | 0.047 |
| Toxicity | Fecundity | 1 | 1.953 | 53.917 | <0.001 |
| | Embryo Lean Weight [mg] | 1 | 1.070 | 64.002 | <0.001 |
| | Reproductive Allocation [%] | 1 | 0.031 | 6.177 | 0.014 |
| Embryonic Stage*SL | Reproductive Allocation [%] | 1 | 0.030 | 5.991 | 0.015 |
| Drainage*Embryonic Stage | Reproductive Allocation [%] | 2 | 0.029 | 5.928 | 0.003 |
| Light Condition*Toxicity | Fecundity | 1 | 0.203 | 5.608 | 0.019 |
| | Embryo Fat Content [%] | 1 | 0.051 | 20.585 | <0.001 |
| | Reproductive Allocation [%] | 1 | 0.076 | 14.490 | <0.001 |

**Table A3.** Discriminant function analysis (DFA) on the life-history traits of female *P. mexicana* from six different populations.

| | Function | | | | |
|---|---|---|---|---|---|
| Life-history trait | 1 | 2 | 3 | 4 | 5 |
| Embryo Lean Weight [mg] | -0.718 | 0.042 | 0.382 | 0.048 | 0.565 |
| Fecundity | 0.631 | 0.094 | 0.079 | -0.105 | 0.302 |
| Female Lean Weight [g] | 0.401 | -0.641 | 0.008 | 0.362 | 0.518 |
| Embryo Fat Content [%] | 0.328 | 0.196 | 0.654 | 0.247 | -0.432 |
| Female Fat Content [%] | 0.107 | 0.511 | -0.354 | 0.638 | 0.198 |
| Reproductive Allotment [%] | 0.075 | 0.269 | 0.420 | -0.316 | 0.492 |
| | | | | | |
| Canonical Correlation | 0.838 | 0.759 | 0.491 | 0.249 | 0.086 |
| Eigenvalue | 2.360 | 1.359 | 0.318 | 0.066 | 0.007 |
| % Variance | 57.4 | 33.1 | 7.7 | 1.6 | 0.2 |
| *Chi*-square | 476.259 | 237.484 | 68.424 | 14.028 | 1.460 |
| *Df* | 30 | 20 | 12 | 6 | 2 |
| *P* | <0.001 | <0.001 | <0.001 | 0.029 | 0.482 |

**Table A4.** Discriminant function analysis (DFA) on the life-history traits of female *P. mexicana* from four different habitat types from southern Mexico.

| | Function | | |
|---|---|---|---|
| Life-history trait | 1 | 2 | 3 |
| Embryo Lean Weight [mg] | -0.682 | 0.243 | 0.259 |
| Fecundity | 0.661 | -0.058 | -0.005 |
| Female Lean Weight [g] | 0.357 | 0.496 | -0.373 |
| Female Fat Content [%] | 0.132 | -0.699 | 0.131 |
| Embryo Fat Content [%] | 0.321 | 0.131 | 0.710 |
| Reproductive Allotment [%] | 0.095 | 0.060 | 0.326 |
| | | | |
| Canonical Correlation | 0.863 | 0.628 | 0.391 |
| Eigenvalue | 2.912 | 0.653 | 0.180 |
| % Variance | 77.8 | 17.4 | 4.8 |
| *Chi*-square | 306.874 | 100.893 | 25.018 |
| *Df* | 18 | 10 | 4 |
| *P* | <0.001 | <0.001 | <0.001 |

**Table A5.** Discriminant function analysis (DFA) ($1^{st}$ function only) on the life-history traits of female *P. mexicana* from (*a*) light versus dark habitats, and (*b*) non–toxic versus toxic habitats.

| Life-history trait | (*a*) | (*b*) |
|---|---|---|
| Fecundity | 0.624 | -0.546 |
| Embryo Lean Weight [mg] | -0.549 | 0.690 |
| Female Lean Weight [g] | 0.444 | -0.097 |
| Embryo Fat Content [%] | 0.409 | -0.159 |
| Reproductive Allotment [%] | 0.134 | -0.030 |
| Female Fat Content [%] | 0.003 | -0.508 |
| | | |
| Canonical Correlation | 0.819 | 0.720 |
| Eigenvalue | 2.042 | 1.075 |
| % Variance | 100.0 | 100.0 |
| *Chi*-square | 169.119 | 110.961 |
| *Df* | 6 | 6 |
| *P* | <0.001 | <0.001 |

# FIGURE LEGENDS

**Figure A1.** Scatter plots of mean embryo dry weight [mg] versus stage of development for *Poecilia mexicana* of (*a*) the Río Grijalva/Usumacinta drainage / SM, (*b*) the Río Tamesí/Pánuco drainage / $NM_2$, and (*c*) the Río Soto la Marina drainage / $NM_1$. Stages are determined on a progressive scale with the earliest (5) being the neurula stage and the oldest (50) being equivalent to ready-to-be-born embryos (after Haynes 1995; Reznick et al. 2002). MI: matrotrophy index.

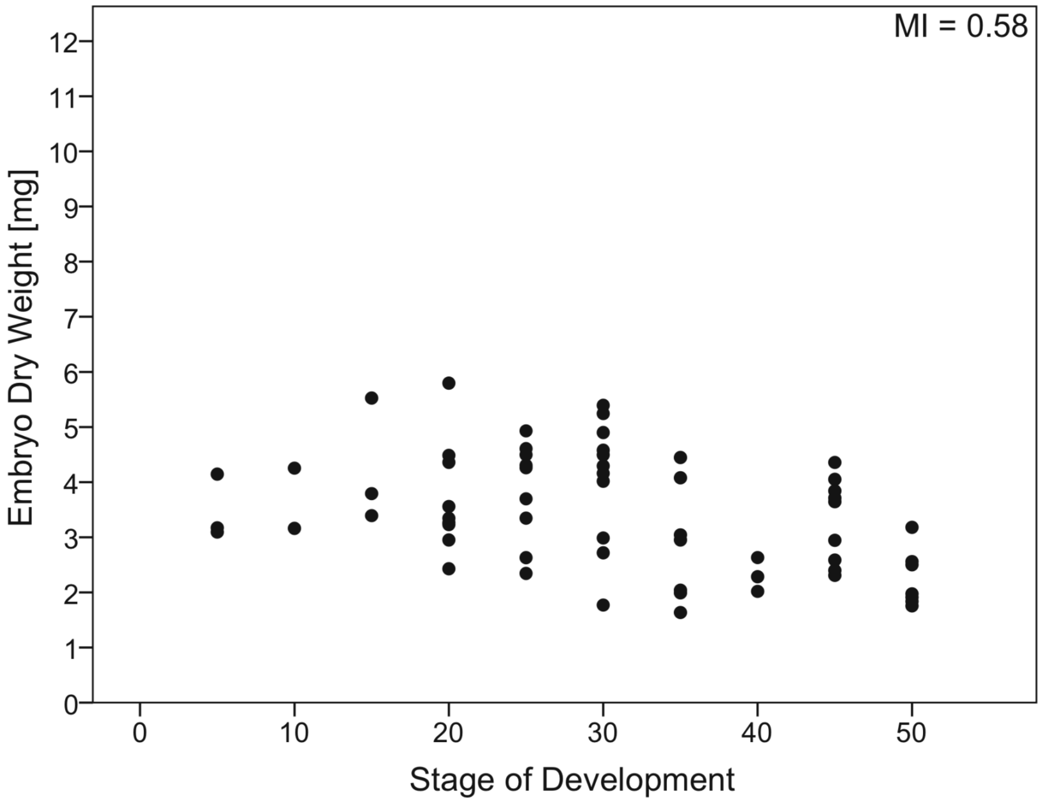
MI = 0.58
Embryo Dry Weight [mg]
0
1
2
3
4
5
6
7
8
9
10
11
12
0
10
20
30
40
50
Stage of Development

Figure A1a

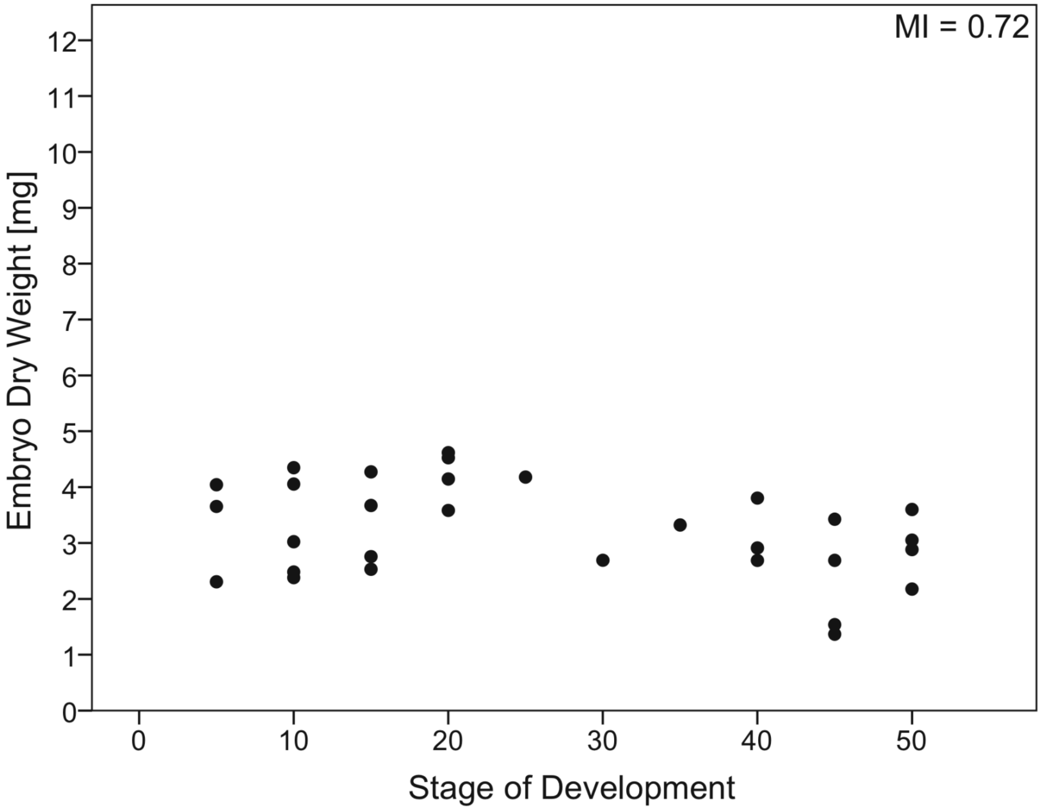
MI = 0.72
Embryo Dry Weight [mg]
0
1
2
3
4
5
6
7
8
9
10
11
12
0
10
20
30
40
50
Stage of Development

Figure A1b

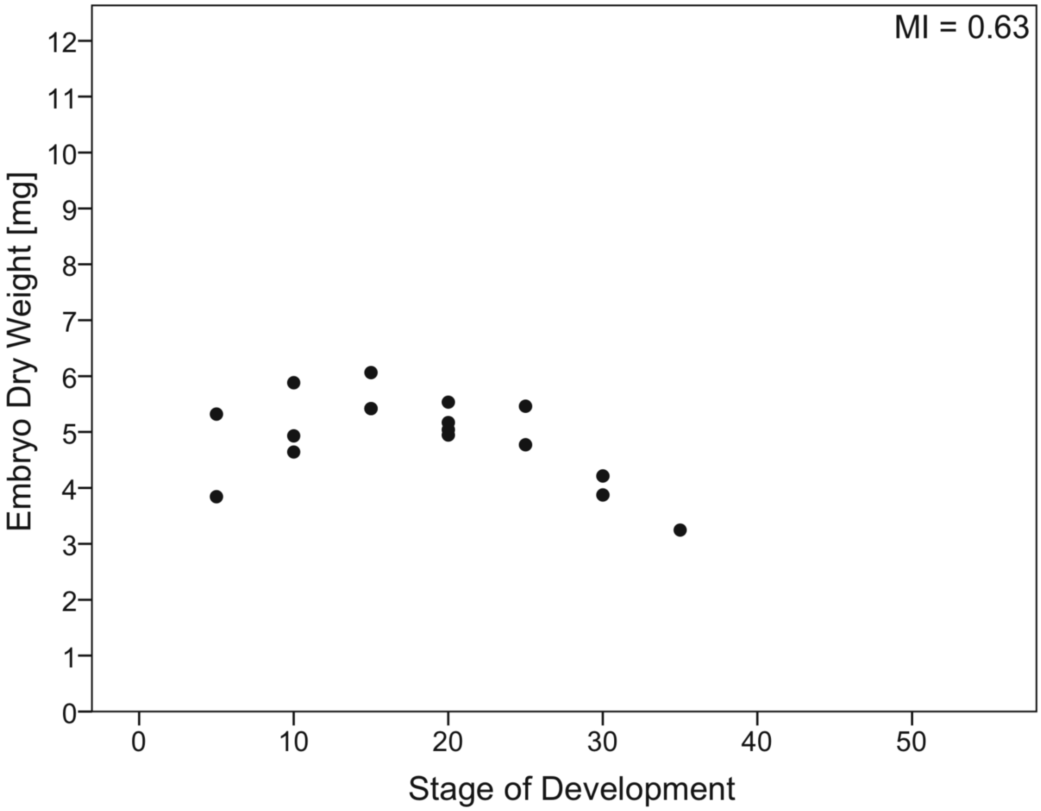
MI = 0.63
Embryo Dry Weight [mg]
0
1
2
3
4
5
6
7
8
9
10
11
12
Stage of Development
0
10
20
30
40
50

Figure A1c

# APPENDIX B

## SUPPLEMENTARY MATERIAL TO CHAPTER 3

Published as online Supplement in 2010, *Naturwissenschaften* **97**:133-141

**Table B1.** Sample locations and sample dates. In the main manuscript, the two sites Arroyo Bonita and Río Amatan are pooled and analyzed together as Río Oxolotán sites. $N$ = ratio of ‘pregnant females to total collected (and dissected) females’.

| Species | Site | Coordinates | $N$ | Sample Date(s) |
|---|---|---|---|---|
| *Poecilia mexicana* | Arroyo Bonita (non-sulfidic) | 17º25’ N, 92º45’ W | 37/43 | June 2007 & January 2008 |
| | Río Amatan (non-sulfidic) | 17º25’ N, 92º47’ W | 25/41 | June 2007 & January 2008 |
| | El Azufre (sulfidic) | 17º26’ N, 92º46’ W | 27/71 | June 2007 & January 2008 |
| *Poecilia sulphuraria* | Baños del Azufre (sulfidic) | 17°34’ N, 93°09’ W | 30/30 | August 2008 & January 2009 |
| *Gambusia sexradiata* | Río Teapao (non-sulfidic) | 17°33' N, 92°57' W | 47/53 | January 2009 |
| | Mogote del Puyacatengo (sulfidic) | 17°35' N, 92°54’ W | 19/40 | January 2009 |
| *Gambusia eurystoma* | Baños del Azufre (sulfidic) | 17°34’ N, 93°09’ W | 27/27 | August 2008 & January 2009 |

**Table B2.** Univariate results from the MANCOVA on female life-histories of four different species of poeciliids from two different genera and three levels of evolutionary exposure to toxicity. SL = standard length. All dependent variables and covariates were log–transformed (or arcsine–transformed if percentages) to accommodate for a potential non–linear relationship between the variables. Only significant results are shown. Adjusted $R^2$ = 0.975.

| Factor | Dependent Variable | *df* | Mean Squares | *F* | *p* |
|---|---|---|---|---|---|
| SL [mm] | Female Lean Weight [g] | 1 | 7.382 | 3197.679 | <0.001 |
| | Fecundity | 1 | 6.172 | 196.487 | <0.001 |
| | Embryo Lean Weight [mg] | 1 | 0.525 | 33.761 | <0.001 |
| | Female Fat Content [%] | 1 | 0.010 | 3.998 | 0.047 |
| | Embryo Fat Content [%] | 1 | 0.041 | 6.714 | 0.010 |
| Embryo Stage | Female Lean Weight [g] | 1 | 0.019 | 8.242 | 0.005 |
| | Embryo Fat Content [%] | 1 | 0.018 | 7.526 | 0.007 |
| Genus | Female Lean Weight [g] | 1 | 0.042 | 18.049 | <0.001 |
| | Fecundity | 1 | 2.597 | 82.677 | <0.001 |
| | Embryo Lean Weight [mg] | 1 | 0.268 | 17.214 | <0.001 |
| | Embryo Fat Content [%] | 1 | 0.034 | 14.462 | <0.001 |
| | Reproductive Allocation [%] | 1 | 0.138 | 22.633 | <0.001 |
| Toxicity-exposure | Female Lean Weight [g] | 2 | 0.068 | 29.643 | <0.001 |
| | Fecundity | 2 | 5.931 | 188.82 | <0.001 |
| | Embryo Lean Weight [mg] | 2 | 2.697 | 173.521 | <0.001 |
| | Female Fat Content [%] | 2 | 0.185 | 29.189 | <0.001 |
| | Reproductive Allocation [%] | 2 | 0.236 | 38.677 | <0.001 |
| Genus*Toxicity-exposure | Female Lean Weight [g] | 2 | 0.046 | 19.995 | <0.001 |
| | Fecundity | 2 | 1.212 | 38.575 | <0.001 |
| | Embryo Lean Weight [mg] | 2 | 1.090 | 70.116 | <0.001 |
| | Female Fat Content [%] | 2 | 0.218 | 34.273 | <0.001 |
| | Embryo Fat Content [%] | 2 | 0.022 | 9.284 | <0.001 |
| | Reproductive Allotment [%] | 2 | 0.077 | 12.594 | <0.001 |
| Error | Female Lean Weight [g] | 204 | 0.002 | | |
| | Fecundity | 204 | 0.031 | | |
| | Embryo Lean Weight [mg] | 204 | 0.016 | | |
| | Female Fat Content [%] | 204 | 0.006 | | |
| | Embryo Fat Content [%] | 204 | 0.002 | | |
| | Reproductive Allotment [%] | 204 | 0.006 | | |

**Table B3.** Structure matrix and test statistics for discriminant function analysis (DFA) on the life-history traits of female poeciliids based on differences in toxicity-exposure and genus.

| | Function | | | | |
|---|---|---|---|---|---|
| Life-history trait | 1 | 2 | 3 | 4 | 5 |
| Embryo Lean Weight [mg] | -0.724 | 0.353 | 0.162 | 0.508 | -0.024 |
| Fecundity | 0.737 | 0.263 | -0.368 | 0.466 | -0.088 |
| Female Lean Weight [g] | 0.021 | -0.765 | 0.009 | 0.399 | 0.120 |
| Embryo Fat Content [%] | -0.023 | 0.101 | 0.133 | -0.690 | 0.646 |
| Female Fat Content [%] | 0.285 | 0.128 | 0.855 | -0.307 | -0.244 |
| Reproductive Allocation [%] | 0.256 | 0.583 | -0.285 | 0.676 | 0.243 |
| | | | | | |
| Canonical Correlation | 0.880 | 0.647 | 0.517 | 0.270 | 0.134 |
| Eigenvalue | 3.434 | 0.718 | 0.364 | 0.078 | 0.018 |
| % Variance | 74.4 | 15.6 | 7.9 | 1.7 | 0.4 |
| *Chi*-square | 499.150 | 193.826 | 82.863 | 19.165 | 3.687 |
| *Df* | 30 | 20 | 12 | 6 | 2 |
| *P* | <0.001 | <0.001 | <0.001 | 0.004 | 0.158 |

**Table B4.** Structure matrices and test statistics for discriminant function analyses (DFA) on the life-history traits of female poeciliids from habitats with different levels of evolutionary exposure to toxicity (left) and toxic versus non-toxic habitats (right).

| | Function | | Function |
|---|---|---|---|
| Life-history trait | 1 | 2 | 1 |
| Embryo Lean Weight [mg] | -0.584 | 0.607 | -0.507 |
| Fecundity | 0.754 | -0.260 | 0.722 |
| Female Lean Weight [g] | -0.259 | -0.463 | -0.268 |
| Female Fat Content [%] | 0.340 | 0.390 | 0.348 |
| Embryo Fat Content [%] | 0.052 | 0.080 | 0.055 |
| Reproductive Allocation [%] | 0.426 | 0.222 | 0.433 |
| | | | |
| Canonical Correlation | 0.775 | 0.555 | 0.774 |
| Eigenvalue | 1.502 | 0.445 | 1.491 |
| % Variance | 77.1 | 22.9 | 100.0 |
| *Chi*-square | 265.430 | 76.021 | 188.949 |
| *Df* | 12 | 5 | 6 |
| *P* | <0.001 | <0.001 | <0.001 |

**Table B5.** Component matrix for the principal components analysis (PCA) on the life-history traits of female poeciliids from three different levels of evolutionary exposure to toxicity and two genera.

| Life-history trait | Component<br>1 | <br>2 |
|---|---|---|
| Female Lean Weight [g] | -0.122 | 0.065 |
| Fecundity | 0.981 | -0.065 |
| Embryo Lean Weight [mg] | -0.695 | -0.530 |
| Female Fat Content [%] | 0.358 | 0.707 |
| Reproductive Allocation [%] | 0.703 | -0.552 |
| Embryo Fat Content [%] | -0.249 | 0.647 |

## FIGURE LEGEND

**Figure B1.** Group centroids ± SDs of discriminant function analyses of female life-history traits based on separation on toxicity exposure alone while controlling for female size (SL) and embryo stage. Nontoxic: *White circle, P. mexicana* and *G. sexradiata*; incipient toxic: *grey circle*, *P. mexicana* and *G. sexradiata*; endemic toxic: *dark grey circle*, *P. sulphuraria* and *G. eurystoma*.

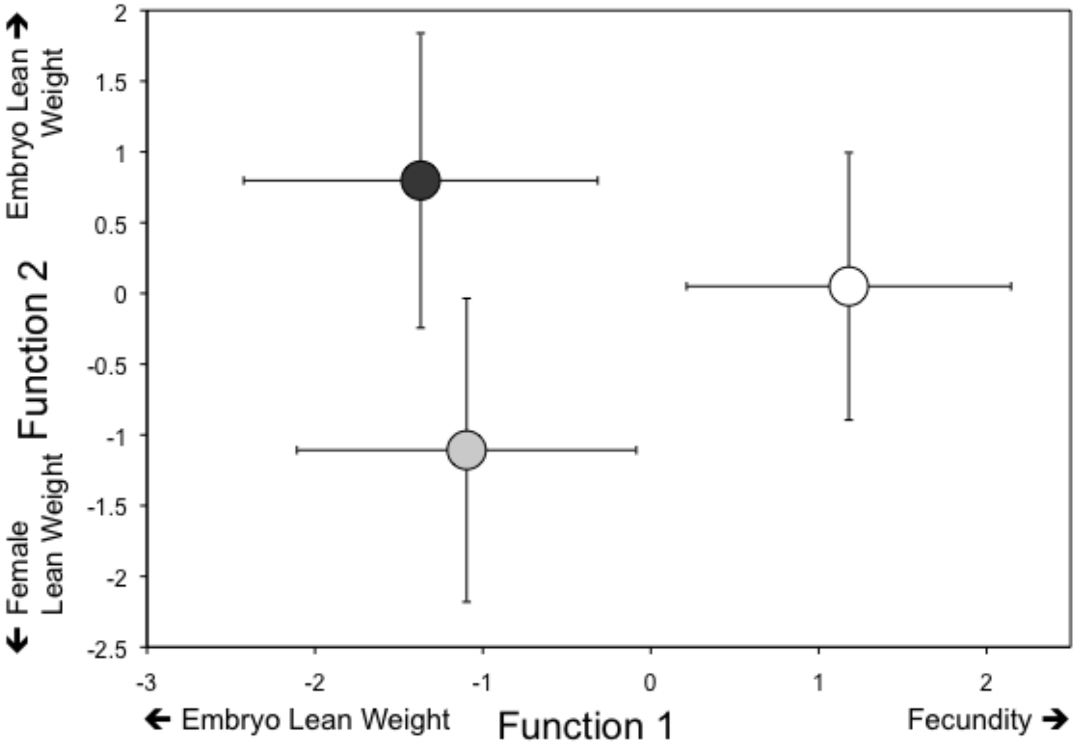
Embryo Lean Weight ➔
Function 2
🡐 Female Lean Weight
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-2
-1
0
1
2
🡐 Embryo Lean Weight
Function 1
Fecundity ➔

Figure B1