

GENETIC DETERMINATION VS. FLEXIBILITY:
HOW PLASTIC ARE BEHAVIORS AND
UNDERLYING PHYSIOLOGICAL MECHANISMS IN A
SYSTEM OF LOCALLY ADAPTED POPULATIONS?

By

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Abstract: An important goal in evolutionary biology is to conceptually incorporate phenotypic plasticity into the framework of trait evolution. Here, I used a system of extremophile fish, *Poecilia mexicana*, locally adapted to all combinations of sulfidic and cave habitats, to test for genetic and plastic variation in energy metabolism and potential links between physiological and behavioral traits. Energy metabolism was predicted by habitat of origin, with cavefish showing higher routine metabolic rates, and sulfidic fish generally showing higher peak metabolic rates, although the extent to which was dependent on resource availability. Together variation in routine and peak metabolic rate lead to complex variation in metabolic scope, which varied depending on the presence of light and sulfide in natural populations as well as resource availability. A combination of resource availability, population of origin, and metabolic physiology (metabolic scope) also predicted male mate choice behavior, which was measured in two different ways: strength of preference for a preferred mate and the number of switches between two potential mates. Strength of preference depended on metabolic scope and the presence of sulfide in the population of origin, with sulfidic fish showing consistently higher strengths of preference, and strength of preference decreasing with metabolic scope overall. The number of switches between stimuli depended on presence of light in the population of origin, food treatment, and metabolic scope. Well-fed *P. mexicana* from cave populations increased their switching behavior with their metabolic scope. These results are part of a larger framework that elucidates the complex interaction between energetics, behavior, their genetic underpinnings, and aspects of the environment that affect the two phenotypes and the relationships between them.

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CHAPTER I

INTRODUCTION

One of the grand challenges in evolutionary biology is the conceptual integration of environmentally induced phenotypic plasticity into our understanding of trait evolution (Pigliucci 2007). Behavior perhaps best epitomizes trait plasticity, as it can be modulated flexibly in response to a variety of environmental cues, and yet many aspects of behavior exhibit clear heritable variation shaped by evolution (Boake 1994). It remains to be tested whether organisms exposed to environmental variation primarily adjust behavioral strategies through plastic responses, evolutionary shifts, or both. For example, curve-billed thrashers (*Toxostoma curvirostre*) show plastic behavioral and physiological responses to variation in food availability (Fokidis et al. 2012). Individuals that consumed inconsistent quantities of food had higher levels of circulating stress hormones, lower body masses, and higher activity levels than conspecifics that were fed consistently, despite the fact that both treatment groups received the same net amount of food over the duration of the study (Fokidis et al. 2012). However, it is unclear whether generations of inconsistent feedings might selectively alter the thrasher behavior, or whether both groups of birds might retain similar genetic behavioral determinants, with both groups continuing to modulate their behavior and physiology plastically. Conversely, laboratory-reared great tits (*Parus major*) exhibit heritable variation in exploratory behavior (Drent et al. 2002), which can have significant consequences for foraging and energy acquisition (Marchetti & Drent 2000). However, the study demonstrating these heritable differences does not address whether food availability or other environmental factors might induce plastic behavioral changes,

or how such plasticity may differ between two heritable behavioral types (bold vs. shy).

Disentangling effects of plasticity and genetic change on behavioral phenotypes is a key goal in evolutionary biology. Creating a complete picture of behavioral determination and flexibility will also require an understanding of the myriad phenotypic traits that influence it and the degree to which they show their own ecologically-mediated plasticity.

Energy availability is one aspect of ecology with profound behavioral implications in a variety of systems, particularly because it can impose constraints that require individuals to trade-off energy allocation to different behaviors and other phenotypic traits (McKnab 2002).

Accordingly, energy-limitation can force individuals to plastically re-prioritize their time budget; for example, three-spined stickleback favor food acquisition over predator avoidance when infected with cestodes that affect their energy consumption (Godin & Sproule 1988).

Conversely, chronically resource-limited environments can also select for behavioral modifications with a strong genetic basis. For example, cave isopods, which are adapted to an environment where food is scarce and unreliably available, diverged behaviorally from their epigeal ancestors, exhibiting more frequent solitary foraging trips rather than gregarious, sit-and-wait strategies (Hüppop 2000). The documented relationships between energy availability and behavior prompt the question whether and how behaviors are linked to metabolic physiology (Careau & Garland 2012; Killen et al. 2013). Like behavior, aspects of metabolic physiology, such as routine and peak metabolic rates as well as metabolic scope, can plastically change based on current resource availability (Killen et al. 2011; Miller et al. 2014) and adaptively evolve in response to ecological sources of selection (Killen et al. 2010; Dijkstra et al. 2013). Energy physiology can also serve as a predictor for behavior in a wide breadth of taxa. For example, common carp characterized by high exploratory behavior are also those with the highest metabolic rates (Jenjan et al. 2013). Dominant prawns, the most successful fighters, had higher resting metabolic rates than their competitors (Brown et al. 2003), and deer mice with high basal metabolic rates were more active in cold weather than conspecifics (Sears et al. 2009).

In this study, I tested for genetic and plastic variation in energy metabolism and illuminated potential links between physiological and behavioral traits. Specifically, I conducted a common garden experiment to perform comparative analyses between closely related populations that live in and have evolved in response to different environmental conditions. I used a system of locally adapted populations (evolutionarily distinct lineages) exposed to differential resource availability (an environmental effect potentially inducing plastic changes in phenotypic traits) along with physiological and behavioral assays to investigate proximate and ultimate causes of variation in male mating behavior.

Atlantic mollies (*Poecilia mexicana*) are livebearing fish that have colonized a variety of habitats with contrasting environmental conditions in Mexico, including toxic, hydrogen sulfide (H_2S) rich springs and caves (Tobler et al. 2006; 2011). Hydrogen sulfide is a naturally occurring chemical present in a variety of aquatic habitats, including deep-sea hydrothermal vents, aquatic sediments, and freshwater sulfide springs (Jannasch & Mottl 1985; Wang & Chapman 1999; Dare & Hubert 2001). Regardless of its environmental source, hydrogen sulfide causes and aggravates hypoxia in aquatic environments (Bagarinao 1992). It also acts as a toxin by blocking the respiratory enzyme cytochrome c oxidase (COX) and hence halting ATP production (Torrans & Clemens 1982; Cooper et al. 2008). At threshold concentrations, hydrogen sulfide reduces fish ventilation rate, inhibits growth and survival, lowers the rate of food consumption and conversion, and impairs both egg production and survival of young (Bagarinao 1992). However, Atlantic mollies that occupy sulfide springs are adapted to the inhospitable conditions. Evolutionarily independent lineages have evolved H_2S -resistant COXs, which reduce sulfide's negative impact on ATP production (Pfenninger et al. 2014), and they have a heritable increase in H_2S detoxification ability through up-regulation of the sulfide:quinone oxidoreductase pathway (Tobler et al. 2014), a process that is energetically costly (Ip et al. 2004; Hildebrandt & Grieshaber 2008). In their natural habitats, mollies also take advantage of the natural gradient present in the water column, where diffused oxygen concentrations are highest and H_2S

concentrations lowest at the water's surface. Sulfide spring mollies spend up to 73% of their time breathing at the water's surface, a behavior known as aquatic surface respiration (ASR), which profoundly affects short-term survival (Plath et al. 2007) but constrains energy acquisition (Tobler et al. 2009). Consequently, living in sulfide springs affects organismal energy budgets, both through costs associated with the maintenance of homeostasis (H_2S detoxification) and reductions in energy uptake.

Other Atlantic molly populations have eschewed open stream habitats and adapted to live in dark caves (Tobler et al. 2008). Cave-dwelling *P. mexicana* show reduced, but fully functional eyes, slender bodies, and reduced pigmentation (Tobler et al. 2008b; Parzefall 2001). Furthermore, cave populations have undergone sensory shifts and are able to rely on non-visual senses for foraging and social interactions in darkness (Plath et al. 2004; 2006b). Due to the lack of photosynthetic primary production, caves are often resource limited (Hüppop 2000), with effects percolating through the entire food web (Gibert & Deharveng 2002), and accordingly cave habitats of *P. mexicana* have an entirely different trophic ecology than surface streams (Tobler 2008; Roach et al. 2011).

Consequently, *P. mexicana* from both sulfidic and cave habitats are energy-limited, albeit for different reasons. While sulfidic mollies may have plenty of available resources, their energy budget is constrained by foraging limitations and by the energetic demands of H_2S detoxification. Conversely, cave mollies may be free to forage unhindered by ASR, but lack access to abundant food. The evolutionary effects of energetic constraints in extremophile mollies is evident in their reduced body condition (Tobler 2008), evolutionary reductions of costly behaviors (Plath & Schlupp 2008), modifications of energetic demands (Passow, paper in review), evolutionary shifts in life history strategies (Riesch et al. 2010; 2014), and the differential expression of genes associated with energy metabolism (Kelley et al. unpublished data).

Here, I focused on four genetically and phenotypically distinct *P. mexicana* populations from the Cueva del Azufre system of the Tacotalpa river, where both environmental factors – i.e.,

the presence or absence of H_2S and light – occur in a natural factorial design (Plath et al. 2007; Tobler 2008b). The four habitat types include a sulfidic cave (Cueva del Azufre; Parzefall 2001), its sulfidic surface outflow (El Azufre; Tobler et al. 2006); a non-sulfidic cave, Cueva Luna Azufre (Tobler et al. 2008a); and a non-sulfidic surface stream Arroyo Bonita (Tobler et al. 2006). By using common garden raised individuals subjected to different food treatments, I tested how variation in resource availability shapes metabolic physiology, both in terms of plastic and heritable differences across populations that have evolved in context of the different environmental conditions in natural habitats, and whether variation in metabolism is correlated with male mating behavior. Specifically, I asked the following questions: (1) How does metabolic scope vary among populations and food treatments? I quantified metabolic scope by measuring oxygen consumption of individual fish at rest (routine metabolic rate) and after physical exercise (peak metabolic rate). Metabolic scope is calculated as the fold change between routine and peak metabolic rate (Wieser & Forstner 1986) and serves as a metric of the energy an individual can mobilize after accounting for routine metabolic processes, thus having important implications for energy budgets and energy allocation to other traits and processes (Killen et al. 2007). I expected both plasticity in response to food availability and evolved population differences, with high food treatment individuals exhibiting higher metabolic scopes overall (Priede 1985) and mollies from energy-limited environments having the greater variability in metabolic scopes between the two food treatments (Komers, 1997). (2) Do aspects of metabolic physiology explain variation in male mating behavior, and are such relationships variable across populations and food treatments? I quantified two metrics of male mating behavior, the strength of a male's preference for a particular female and the number switches between two female stimuli. I predicted that both behavioral measurements would depend on metabolic scope, with individuals having a high metabolic scope sharing time more equally between females (i.e., having a lower strength of preference) and switching between females more frequently. Alternatively, metabolic scope may be predictive of mate choice behavior only when resources are scarce, but show no significant

correlation in the high food treatment (Killen et al. 2013). I also expected more behavioral plasticity in males adapted to the energy-stressed sulfidic and cave environments (Komers 1997), which should be manifested in significant interaction terms between variables describing environmental conditions in the habitats of origin (sulfide/cave) and variables describing internal (metabolic scope) and external (food treatment) energy availability.

CHAPTER II

MATERIALS AND METHODS

Study populations and food treatments

I used common garden raised fish from four populations living under different environmental conditions in the Tacotalpa river drainage: a sulfidic cave (Cueva del Azufre), non-sulfidic cave (Cueva Luna Azufre), sulfidic surface stream (El Azufre), and non-sulfidic surface stream (Arroyo Bonita; see Tobler et al. 2008). All fish were originally raised in mixed-sex, randomly outbred stock populations in non-sulfidic water, kept on a 12:12 light:dark cycle, and fed *ad libitum* twice a day.

To manipulate male condition, I arranged haphazardly selected adult males from each population in a series of 40-liter tanks. Five males and one female of a single population occupied each tank, which was assigned to either a high or low food treatment (15 males/treatment/population). The amount of food for the high food treatment (FH, in grams) was calculated as follows: $FH = 0.0125 * (\text{total fish mass per tank})^{0.65}$. I used the scaling coefficient described by Calder III (1987) as a predictor of mass-dependent energy requirements for freshwater fish. The low food treatment (FL) was half the amount of food given in the high food treatment. All fish were fed a varietal fish flake food (American Brine Shrimp Company, Ogden, UT). Fish were fed twice a day Monday through Friday and once daily on weekends for the trial's duration. Treatments lasted at least 21 days before behavioral and physiological experiments began.

Metabolic physiology

To test for variation in aspects of metabolic physiology among food treatments and populations, I measured the peak and then routine metabolic rates (PMR and RMR, respectively) of males after each mate choice trial using a closed chamber respirometry system (Steffensen 1989). All MR measurements were taken on post-absorptive fish (Norin & Malte 2011; Timmerman & Chapman 2004b). PMR is a measurement of an organism at maximum exertion, and I achieved this by agitating fish into burst swimming performance for five minutes (Cutts et al. 2002). In other fish species, such quick-start burst swimming has been shown to be more metabolically demanding than sustained swimming (Puckett & Dill 1984). After chase trials, oxygen consumption of individual fish was immediately measured for a 30-minute period (see below for detailed procedures). After PMR trials, males remained isolated in respirometry chambers and were allowed to rest undisturbed for 12 hours in water continuously oxygenated through an air stone (Cutts et. al, 2002). Prior to the start of RMR testing, the chamber was flushed with fresh, aerated water to remove metabolic waste product (Timmerman & Chapman, 2004a). Oxygen consumption measurement was then repeated to obtain an estimate of RMR. After measuring both PMR and RMR, I weighed and measured the standard length of each male before returning the subjects to stock lab tanks.

General procedures for the measurement of oxygen consumption were identical for both PMR and RMR trials and followed Passow et al. (submitted). In short, individual males were placed into a black respirometry bottle (580 ml volume) filled completely with aerated water. The chamber was then sealed, and a YSI ProODO probe (YSI Inc., Yellow Springs, OH), which simultaneously measures dissolved oxygen concentration and temperature at 10-second intervals, was inserted through an opening in the bottle cap. The entire system was sealed with plumber's putty to prevent gas diffusion. Respirometry bottles were arranged in a one-inch water bath to minimize temperature fluctuation. All trials took place at ambient temperatures averaging between 19 - 25 °C.

Raw data of metabolic rate measurements represented oxygen concentrations through time. PMR and RMR were then calculated as the slope of a regression with time (in hours) as the independent variable and oxygen concentration (in mg/l) as the dependent variable. To correct for the volume of water in each container, all values were multiplied by the respiratory volume, such that MR measurements are provided in mgO₂/hr. For PMR, only the first 30 minutes of data were used for the regression. For RMR, I eliminated the first 90 minutes of recorded data from analysis to ensure males had recovered from the disturbance of the trial set-up. Regressions were then calculated based on the subsequent 347 minutes of data. Finally, factorial metabolic scope was calculated as the ratio of PMR to RMR (Wieser & Forstner 1986).

All metabolic rate data were first mass corrected by calculating the residuals of a regression between body mass and metabolic rate (both log₁₀-transformed). Mass-specific metabolic rate measurements as well as metabolic scope were then used as dependent variables in analyses of variance (ANOVA), including presence of light in the natural habitats (cave vs. surface), presence of sulfide in the natural habitats (sulfidic vs. non-sulfidic), and food treatment as independent variables. Temperature was not included in analysis, as it was not a significant predictor of scope. All ANOVA models were first built fully factorial, but non-significant ($p > 0.05$) interaction terms were subsequently eliminated in a step-wise fashion. All datasets fulfilled the assumptions of normal distribution of data and constant variance.

Behavioral trials

To test whether and how resource availability, habitat of origin, and metabolic scope affects male mating strategies, I used a binary mate choice experiment to quantify two aspects of male mating behavior, including the propensity of a male to spend time with a single female [as opposed to interacting with both female stimuli equally; strength of preference (SOP)] and the propensity for exploratory behavior (i.e., the number of switches between preference zones). Binary mate choice trials were conducted in a 200-liter choice tank filled two thirds with de-chlorinated and aerated

water. The tank was divided into three equal areas with markings on the front: a central neutral zone and two preference zones at either end. Each preference zone contained a mesh cylinder for holding stimulus females, allowing for passage of both visual and chemical cues to focal males (see Plath et al. 2005; Tobler et al. 2011 for similar experimental setups). Females were allowed to acclimate for three minutes, after which a focal male was introduced to the center of the tank. Upon introduction of the male to the tank, I immediately started recording the behavioral trial using a Sony video camera (Model HDR-XR260) mounted on a tripod stand to avoid altering behavior via disturbance from a human observer. After 15 minutes, the position of the two females was switched, and male behavior was recorded for an additional 15 minutes. Switching the position of the female stimuli was used to eliminate potential side biases (Landmann et al. 1999), such that the time a male spent in a preference zone truly indicated association with a particular female rather than an unrelated propensity to stay on a certain side of the tank. Males that did not switch between females and showed a low strength of preference ($SOP \leq 0.059$, switches = 0) were eliminated from all analyses. These males showed very low activity, and the weak strength of preference was due to a failure of the male to leave a particular preference zone even as females were switched, rather than an active choice to divide time equally between females (Landmann et al. 1999).

To extract behavioral data from mate choice videos, I used the BORIS (Behavioral Observation Research Interactive Software; <http://penelope.unito.it/boris>) video scoring software. Behavioral scoring started when focal males started to swim freely in the choice tank (Plath et al. 2005), and I recorded time spent in the preference zone of each female. Association time is a standard measure for mate choice in poeciliid fishes (McLennan & Ryan 1997; Bisazza et al. 2001; Kahn et al. 2009; Verzijden et al. 2012; Plath et al. 2013). In poeciliids, association times are repeatable preference metrics (Cummings & Mollaghan 2006) and are predictive of actual reproductive outcomes (Plath et al. 2006b; Walling et al. 2010). I stopped behavioral measurements after ten minutes and did not resume measurements until after the video showed

the female switch and the male again resumed swimming. At this point, the preference zones for the two females were reversed (i.e. all time spent in the rightmost preference zone is attributed to female “A” before the switch, and then to female “B” afterward). I again monitored association behavior for ten minutes. Any male who did not move within the first five minutes either pre- or post-switch was eliminated from analysis ($n=3$), as each segment of the trial was a total of 15 minutes long, including acclimation time, and behaviors were analyzed for exactly ten minutes per segment. Based on male association time with each female, I calculated SOP as $(\text{time with preferred female} - \text{time with unpreferred female}) / (\text{time with preferred female} + \text{time with unpreferred female})$. Hence, SOP in the context served as a measurement of the male’s propensity to spend all of his time with a single female ($\text{SOP}=1$) vs. splitting time evenly between two potential mates ($\text{SOP}=0$). I also recorded the number of switches made from one female to another as a measurement of the male’s capacity for and interest in trading-off time between females.

Behavioral data were analyzed using analysis of covariance (ANCOVA) with SOP (arc-sine-square-root-transformed) and the number of switches (square-root-transformed) as dependent variables. The presence of light in the natural habitats (cave vs. surface), presence of sulfide in the natural habitats (sulfidic vs. non-sulfidic), and food treatment served as independent variables, and metabolic scope as a covariate. Like in physiological analyses, models were first run fully factorial, and non-significant terms ($p > 0.05$) were eliminated in a step-wise fashion. Although RMR and PMR could also have served as potential covariates, I focused on metabolic scope for behavioral analyses, because it represents a measurement of the energy an organism can mobilize after accounting for its most basic physiological needs; i.e, it is essentially a disposable energetic currency (Killen et al. 2007; Priede 1985). Furthermore, a model selection approach also indicated that metabolic scope is the most reliable physiological predictor of behavioral variation (results not shown).

CHAPTER III

RESULTS

My first goal was to determine whether there was variation in energy metabolism among populations and how each population responded to food treatments. To look for population effects, I used sulfide and cave as independent variables describing the environmental conditions to which these mollies had adapted on an evolutionary timescale. Metabolic scope variation was explained by a significant three-way interaction between sulfide, cave, and food treatment, as well as significant sulfide and sulfide by cave treatment effects (**Table 1a**). Overall, fish from non-sulfidic surface habitats had the lowest metabolic scope (in both food treatments), fish from the sulfidic surface habitats exhibited the highest metabolic scope (particularly in the low food treatment; **Figure 1a**), and the two cave populations were intermediate. Plasticity in metabolic scope (i.e., differences among food treatments) was only pronounced in the sulfidic surface population.

I then analyzed the two components of metabolic scope separately in order to parse out the seemingly idiosyncratic among population variation in metabolic scope and its plasticity. RMR varied significantly between cave and surface populations (**Table 1b**; $p = 0.043$), with cave fish exhibiting higher RMR than surface fish, irrespective of whether they were derived from sulfidic or non-sulfidic populations (**Figure 1b**). In contrast, PMR was predicted by a 3-way interaction between sulfide, cave, and food treatment, which were in part driven by a pronounced differences between sulfidic and non-sulfidic populations (**Table 1c**). While sulfidic fish showed higher PMR than those from non-sulfidic populations, the three-way interaction appears to be

Table 1: ANOVA results showing the effects of sulfide, cave, and food on a) metabolic scope, b) routine metabolic rate, and c) peak metabolic rate. Bold values indicate significance at $P < 0.05$.

Source	<i>df</i>	<i>F</i>	<i>P</i>	Partial Eta Squared
<u>a) Metabolic scope</u>				
Sulfide	1,73	8.470	0.005	0.104
Cave	1,73	0.002	0.967	<0.001
Food	1,73	1.485	0.227	0.020
Sulfide*Cave	1,73	11.038	0.001	0.131
Sulfide*Food	1,73	0.693	0.408	0.009
Cave*Food	1,73	0.751	0.389	0.010
Sulfide*Cave*Food	1,73	4.589	0.036	0.059
<u>b) Routine metabolic rate</u>				
Sulfide	1,77	2.027	0.159	0.026
Cave	1,77	4.247	0.043	0.052
Food	1,77	0.683	0.411	0.009
<u>c) Peak metabolic rate</u>				
Sulfide	1,73	17.024	0.000	0.189
Cave	1,73	4.333	0.041	0.056
Food	1,73	0.233	0.631	0.003
Sulfide*Cave	1,73	1.810	0.183	0.024
Sulfide*Food	1,73	0.023	0.879	<0.001
Cave*Food	1,73	1.975	0.164	0.026
Sulfide*Cave*Food	1,73	4.762	0.032	0.061

caused by cavefish in the low food treatment exhibiting similar PMR regardless of whether the populations are sulfidic or non-sulfidic (**Figure 1c**). Consequently, evolution in cave environments appears to primarily drive variation in RMR, and evolution in sulfidic environments variation in PMR, which in combination give rise to the complex population-level variation in metabolic scope.

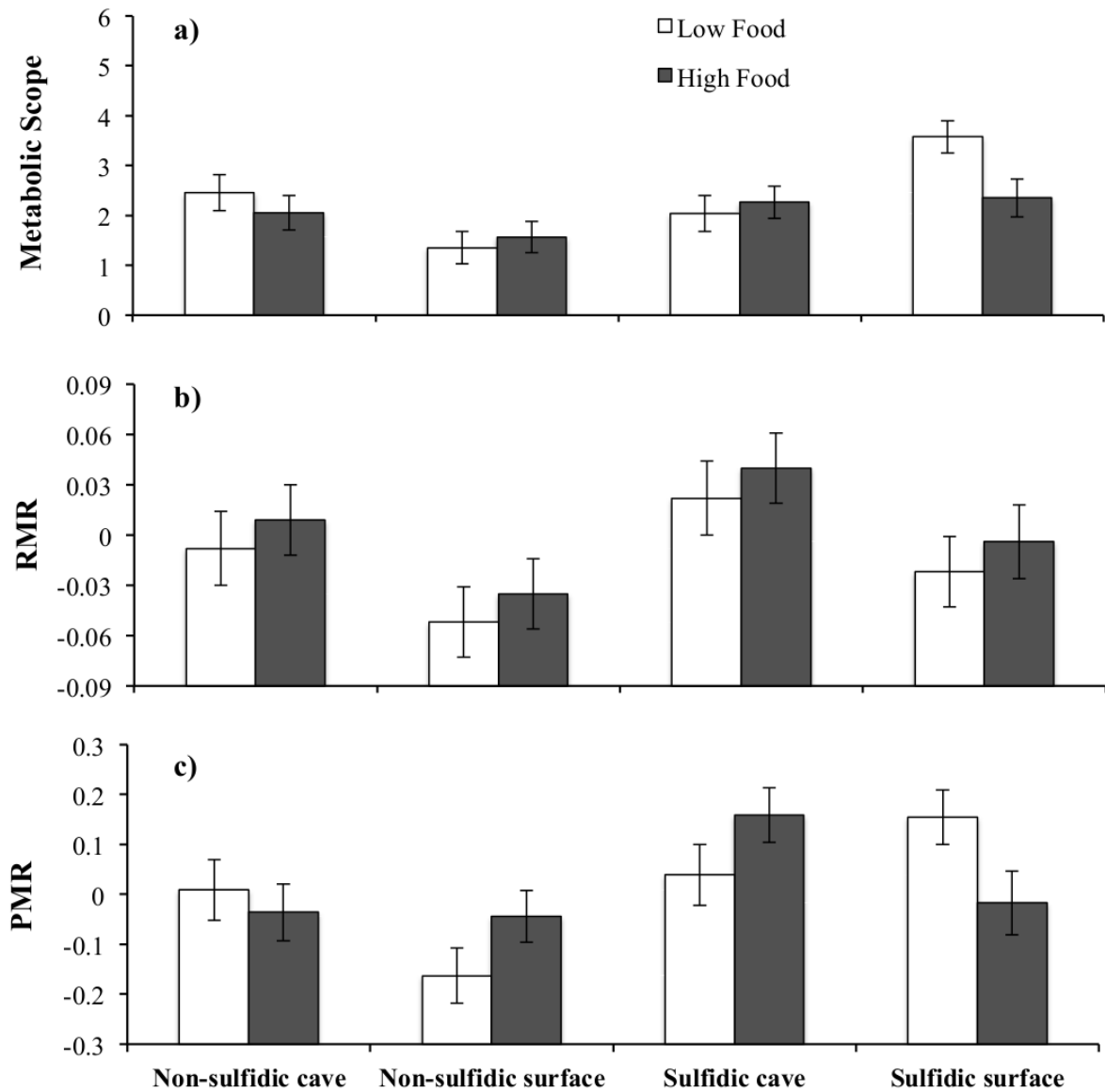


Figure 1: The effects of sulfide, cave, and food treatment on a) metabolic scope, b) routine metabolic rate, and c) peak metabolic rate. Values represent estimated marginal means (\pm standard error).

Behavioral variation and its relation to energy physiology

Metabolic scope and sulfide were both significant predictors of strength of preference (**Table 2**).

All populations show a decrease in the strength of preference with increasing metabolic scopes, and males from sulfidic populations show a consistently higher strength of preference than males from non-sulfidic populations (**Figure 2**).

Table 2: ANCOVA showing the effects of sulfide, cave, and food on strength of preference. Bold values indicate significance at $P < 0.05$. All factors have one degree of freedom

Source	<i>df</i>	<i>F</i>	<i>P</i>	Partial Eta Squared
Sulfide	1,76	4.707	0.033	0.058
Cave	1,76	0.112	0.739	0.001
Food	1,76	0.564	0.455	0.007
Scope	1,76	9.428	0.003	0.110

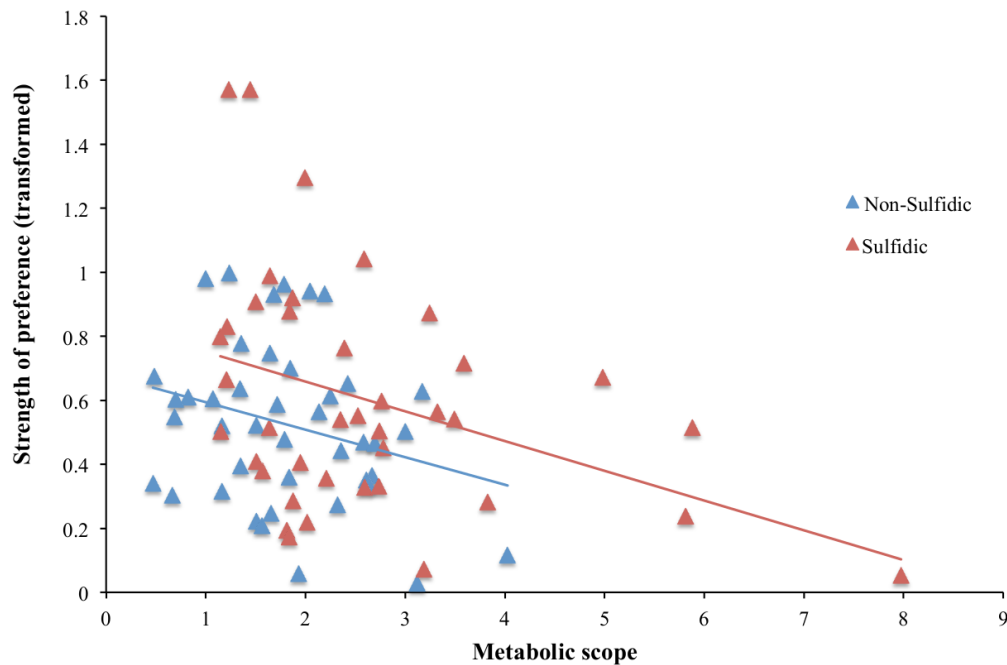


Figure 2: The effects of sulfide and metabolic scope on strength of preference.

Switching behavior depended on metabolic scope, and an interaction between food treatment and cave (**Table 3**). Males with higher metabolic scope tended to switch more frequently between the two females (**Figure 3**). Regardless of scope, cavefish in the high food treatment switched significantly more often than surface fish, and this difference was non-significant in the low food treatment (**Figure 3**). An interaction between light and scope was barely non-significant ($p = 0.053$) and indicated a trend toward increased switching with higher scopes in cavefish, but not in surface fish (**Figure 3**).

Table 3: ANCOVA results showing the effects of sulfide, cave, food, and metabolic scope on switching behavior. Bold values indicate significance at $P < 0.05$. All factors have one degree of freedom.

Source	<i>df</i>	<i>F</i>	<i>P</i>	Partial Eta Squared
Sulfide	1,70	0.058	0.811	0.001
Cave	1,70	1.366	0.246	0.019
Food	1,70	1.078	0.303	0.015
Scope	1,70	4.130	0.046	0.056
Cave*Food	1,70	4.689	0.034	0.063
Food*Scope	1,70	0.069	0.793	0.001
Sulfide*Food	1,70	0.599	0.442	0.008
Cave*Scope	1,70	3.868	0.053	0.052
Sulfide*Cave	1,70	0.874	0.353	0.012
Sulfide*Scope	1,70	0.011	0.917	<0.001

Interestingly, male mate choice did not depend on female size, and strength of preference was not higher for larger females, as has been demonstrated in previous mate choice studies (Plath et. al, 2005, 2006b).

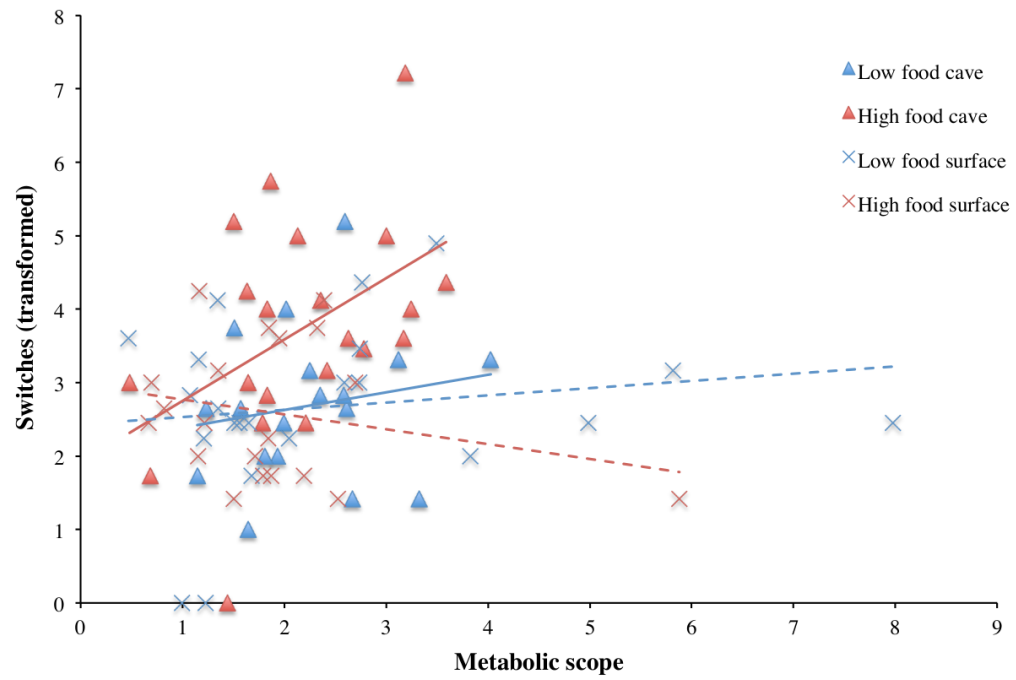


Figure 3: The interactive effects of food treatment, cave, and metabolic scope on switching behavior.

CHAPTER IV

DISCUSSION

In Southern Mexico, *Poecilia mexicana* has colonized different habitat types characterized by the presence and absence of light and toxic hydrogen sulfide. This study uncovered differences in multiple aspects of energy physiology and its plasticity, with populations from cave habitats generally having an elevated routine metabolic rate and populations from sulfidic habitats having modifications in peak metabolic rates and its plastic response to different food treatments. Furthermore, energetic state – estimated through metabolic scope – and population of origin significantly predicted aspects of male behavior. Because all individuals used in this study were common garden raised, significant population differences are indicative of evolved and genetically based phenotypic differentiation both for physiological and behavioral traits, although experimentally induced resource availability also induced plastic changes that in part varied across populations (i.e., evolutionary differences in plasticity). Overall, these results are consistent with previous studies that have documented heritable trait divergence and strong patterns of local adaptation in this system (e.g., Passow et al. submitted; Riesch et al. 2010; Tobler et al. 2008b).

Variation in energy physiology

Variables describing the environmental conditions in the habitats of origin of the investigated populations were the most important predictors of metabolic physiology, indicating genetic variation in metabolism and its plasticity. For metabolic scope, only the population from the

sulfidic surface habitat showed a significant plastic response to food treatment. RMR was an aspect of energy physiology entirely determined by whether populations originated from cave or surface habitats, and PMR was largely predicted by the presence of sulfide in natural habitats, although there was among population variation in how food treatment affected PMR plastically.

The primary productivity of an organism's environment of origin is an important factor in determining routine metabolic rate, with organisms from low productivity environments having lower routine metabolic rates (Mueller & Diamond 2001). For this reason, the elevated RMR documented in cave populations of *P. mexicana* is counterintuitive. Several non-mutually exclusive hypotheses may explain this unexpected result: (1) Reducing metabolism is not the only way to limit energy expenditure; reduction in body size can achieve the same energy economy. Indeed, Passow et al. (submitted) found significant reductions in the body size of cave mollies. However, reducing body size, while lowering energy costs overall, can come at a metabolic expense, because small individuals typically have a larger mass-specific metabolic rate than large ones (Brown et al. 2004; Kleiber 1932). Hence, elevated RMR in cavefish may be a by-product of body size reduction. (2) Caves may not be as nutrient poor as previously assumed. Resource availability, while likely to be limited, is relatively stable over time in tropical caves due to reduced seasonality (Hüppop 2000). Thus, cave populations of *P. mexicana* may have adapted to consistent energy shortages rather than temporal bursts. In the Cueva del Azufre system, continuous food supply is further facilitated by bat colonies depositing guano (in both caves) and by the chemoautotrophic primary production of sulfide oxidizing bacteria (in the sulfidic cave; Roach et al. 2011). (3) Adaptation to cave environments may be energetically costly. Perhaps certain sensory shifts necessitated by life in darkness also demand a higher routine metabolic rate via maintenance costs, as *P. mexicana* from cave habitats have a hyper-developed cephalic lateral line system and a higher taste bud count (Walters & Walters 1965; Parzefall 2001). Furthermore, reduced metabolic rates in response to energy limitation are often a mechanism that preserves stored energy reserves (O'Connor et al. 2000). Because cavefish maintain consistently high

RMRs, even under food stress, they may be trading-off the availability of readily available vs. stored energy, which would explain their poor condition in the wild (Tobler 2008). If they have vital adaptations facilitating cave life with high operating costs, they should resort to the loss of lipid stores in the face of low resource availability rather than reducing routine metabolic processes required to maintain basic physiological processes. One could test this hypothesis by measuring the lipid content relative to body size of cave and surface fish before and after periods of starvation.

Sulfidic mollies may show increased metabolic peaks as a way of allowing for both the up regulation of detoxification pathways and the devotion of time to ASR if required in the presence of H₂S (Tobler et al. 2009). These adaptations are unlikely to incur a baseline maintenance cost via increased RMR, but there must be room for them in the energy budget in a food-independent way via increased PMR. In snapper exposed to hypoxic conditions, metabolic peak was the most important factor in determining tolerance to reduced oxygen. Snappers with higher PMR (and metabolic scopes) were better able to handle extreme hypoxia (Cook et al. 2011). Consequently, future studies should test whether sulfidic mollies tested in sulfidic water show increased RMR and decreased metabolic scope, because a portion of their energy budget is devoted to detoxification and sulfide avoidance.

Previous studies in fishes have primarily tried to elucidate the role of plasticity vs. genetic variation in shaping metabolic rate plasticity. Adaptive plasticity allows organisms to adjust relevant phenotypes to maximize fitness in variable environments (Pigliucci 2005; Zhaoa & Caoa 2009). Hence, plasticity can evolve following the colonization of novel or extreme environments (Lee et al. 2011), or it may be absent or reduced in certain environments (Merila et al. 2004), as it can impose fitness costs under certain environmental conditions (Pigliucci 2005). While many fish taxa demonstrate measurable plasticity in metabolic physiology in response to resource limitation (O'Connor et al. 2000), some exhibit very narrow reaction norms in metabolic rates in the face of experimentally manipulated food deprivation (Rios et al. 2002). Divergent

environments can have evolutionary impacts on active metabolism as well. Two different cichlid species showed varying levels of metabolic efficiency – one species (*P. nyererei*) showed lower levels of oxygen consumption per unit of aggressive behavior than its counterpart (*P. pundamilia*) despite being the more aggressive of the two sister species (Dijkstra et al. 2013). This indicates that metabolic efficiency may allow for more aggressive displays and higher activity levels in general. In the system investigated here, the question is not about whether plasticity or genetic differentiation drive variation in metabolic physiology, because the results indicate they both play a role, and there is even among population variation in plasticity. This is particularly noteworthy considering the close evolutionary relationships among all populations investigated and highlights the mechanistic complexity giving rise to variation in metabolic physiology, even when broad scale studies find striking similarities in overall patterns of metabolic variation (Brown et al. 2004).

Variation in behavior and its relation to metabolic scope

The results of this study indicate that energy physiology can serve as a significant predictor of behavior. The presence of sulfide and metabolic scope independently predicted a male's affinity to spend time with the preferred female. Sulfidic fish showed consistently higher strengths of preference across all scopes, and strength of preference consistently declined with increasing metabolic scope. This is an intuitive finding if one thinks of strength of preference as a willingness to stay with one female, rather than dividing time evenly between two potential mates. The inverse correlation between metabolic scope and strength of preference follows the theoretical performance model relationship between metabolism and activity, where physiology (here, metabolic scope) acts as an indicator of available energy, which in turn dictates an organism's capacity for activity (Careau et al. 2008). Therefore there is a predicted positive relationship between activity levels and metabolic scope (Careau & Garland 2012). For all male

mollies, those with the highest amount of energy to mobilize (highest metabolic scopes) were those that split their time between two mates rather than choosing one and staying put.

Simultaneously, sulfidic mollies – at any given scope – showed higher strengths of preference than their non-sulfidic counterparts, indicating that they were more likely to stick with a preferred female. This may be an evolutionary consequence of the adaptive mechanisms allowing for life in sulfidic environments. It is tempting to speculate that sulfidic mollies may have reduced costly behaviors and stay with a preferred female, because they have a non-plastic section of their energy budget carved out for either up-regulation of sulfur-detoxifying pathways or ASR, should either activity become necessary for survival (Tobler et al. 2014; Plath et al. 2007). Living in the presence of a physiochemical stressor means that maintenance of homeostasis necessitates expensive investments in physiological, morphological, or behavioral coping strategies (Calow 1989; Parsons 1996; Sibly & Calow 1989), before energy can be allocate to other functions (Priede 1985).

Regardless of the proximate cause, I found population-level differences in strength of preference, indicating genetic divergence in this specific male behavior across populations. This is part of a well-documented history of physiological and behavioral divergence between sulfidic mollies and their non-sulfidic ancestors (Tobler et al. 2008b). However, especially striking is the fact that strength of preference did not depend on immediate resource availability for males from any environment. I expected a higher degree of plasticity in sulfidic and cave populations (Komers 1997), which are both energy limited for unique reasons, but strength of preference was completely food independent. This indicates that individual metabolic differences are better predictors of this behavioral metric than food availability.

Switching behavior depended on a mixture of physiology, food treatment, and the absence of light in natural environments. Cave males with higher metabolic scopes switched more often between the two females, investing energy to move back and forth between two potential mates. While this may be an indication of male promiscuity, it is also possible that it represents a

different behavioral syndrome related to exploratory behavior, such as a bold *vs.* shy personality type (Drent et al. 2002). Bold exploratory behavior is often linked to higher resting metabolic rates (Jenjan et al. 2013), and data presented here suggest that metabolic scope may also be a predictor of exploration propensity. To determine whether switching behavior is a metric for promiscuity or for boldness, future experiments will need to correlate switching behavior with copulation attempts and examine exploratory behavior in absence of potential mates.

I was also curious whether and how food deprivation might change the strength of the correlation between metabolic scope and the two aspects of behavior I measured. Because different traits within an individual can show different norms of reaction to an environmental stressor (food deprivation in this case), the presence of a stressor can strengthen or weaken the correlation between the different intra-individual traits (Killen et al., 2013). For example, metabolic scope may be a consistently variable trait between individuals, but behavior may only start to show significant variation in the face of resource limitation as individuals differ in their ability to cope with food-related stress. At this point, metabolic scope may start to become an important physiological correlate to behavior, a trend not seen when food is plentiful. If this were true, not only would the stress of food deprivation alter metabolic scope and behavior individually, it would alter the way in which they mediate, and are therefore predictive of one another. However, I did not find any evidence for an interaction between metabolic scope and food treatments explaining variation in strength of preference or the number of switches. This indicates that the norm of reaction to food deprivation was similar for metabolic scope, strength of preference, and switching behavior within each individual.

Conclusions

I summarized the complex interactions I discovered between evolutionary history, current resource availability, energy physiology, and behavior in **Figure 4**. There are clear differences across populations in metabolic physiology, and different aspects of physiology are affected

depending on the habitat of origin. There are also links between energy physiology and behavior that vary either based on (1) genetic differences between fish from divergent populations, or (2) resource availability inducing plastic differences, based on the behavioral metric being investigated. Therefore, aspects of reproductive behavior depend both on genetic divergence and current environmental variables, and are mediated by energy metabolism.

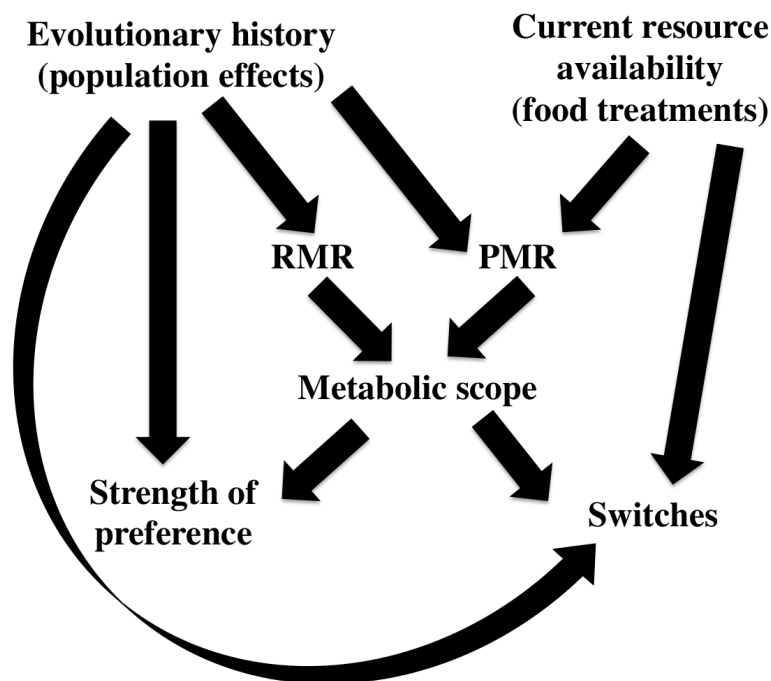


Figure 4: A qualitative summary of documented relationships between population of origin, food treatment, metabolic physiology, and behavior in different populations of *Poecilia mexicana*.

Future studies should explore the link between metabolic physiology and other measurements of behavior. Because life in cave and sulfidic environments requires a carefully balanced energy budget (Bagarinao 1992; Tobler 2008), metabolic physiology should have important implications for any energetically expensive behavior including the reaction to stressful

stimuli (Careau et al. 2008), aggression (Dijkstra et al. 2013), or latency to explore novel environments (Jenjan et al. 2013). These behaviors could also be influenced plastically by any aspect of the environment, not to mention the fact that the behaviorally-predictive power of metabolic physiology can alter in the presence of a stressor (Killen et al. 2013). Improving our understanding of behavior will, in part, involve disentangling the complex interaction between energetics, behavior, their genetic underpinnings, and all aspects of the environment that affect the two phenotypes and the relationships between them.

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VITA

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