DEVELOPMENTAL ENVIRONMENT ALTERS CONDITIONAL AGGRESSION IN

ZEBRAFISH

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Chris Marks

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ZEBRAFISH

Chris Marks

Thesis

Approved: Accepted:

_____   _____
Advisor   Dean of College
Dr. Francisco Moore   Dr. Ronald F. Levant

_____   _____
Committee Member   Dean of the Graduate School
Dr. Stephen C. Weeks   Dr. George R. Newkome

_____   _____
Committee Member   Date
Dr. Brian Bagatto

_____
Department Chair
Dr. Bruce Cushing

ABSTRACT

The developmental environment influences a wide variety of phenotypic traits in the adults of many vertebrates (i.e., developmental plasticity). In this study, we test to see if developmental environment (E_{DEV}) interacts with the adult behavioral environment (E_{BEHAV}) in determining behavioral phenotypes. We reared Zebrafish (Danio rerio) from eggs in either continuously hypoxic or normoxic conditions. We then tested aggression and avoidance (i.e., hiding) levels of fish from each developmental treatment in both environments. Developmental environment was a significant source of variation in avoidance behavior while the stimulus environment did not influence avoidance. Without a period of acclimation we found that E_{BEHAV} and an E_{DEV} X E_{BEHAV} interaction were both significant sources of variation. However, when the fish were allowed to physiologically acclimate to the environment for 16 h, aggression level was highest for fish tested in the environment in which they developed. In that case the E_{DEV} X E_{BEHAV} interaction was the only significant source of variation. These results demonstrate that a more complete understanding of phenotypic response can be gained by incorporating environmental conditions across multiple time scales.

ACKNOWLEDGEMENTS

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CHAPTER I

BACKGROUND

Classic quantitative genetic models divide the fundamental factors influencing

phenotypic variation into genetic and environmental (Falconer, 1981; Pigliucci, 2001;

Dewitt and Scheiner, 2004b). Both of these factors as well as an interaction between the

two can be important contributors to variation in a given trait. The genetic sources of

variation can be further divided into additive and non-additive genetic components

(Falconer, 1981). Additive genetic variation is the result of the independent action of

genes on trait value. Non-additive genetic variation is created primarily by genetic

interactions. While gene-gene and gene-environment interactions are increasingly

recognized as important in evolution (Whitlock et al., 1995; Scheiner and Dewitt, 2004),

interactions between ontogenetic (developmental) environments have largely been

ignored and are also potentially important evolutionary influences (Pigliucci, 2001; Sih,

2004). In this study, we test for additive and interaction effects of developmental and

adult environments in Zebrafish (*Danio rerio*) behavior.

Environmental influence on traits (plasticity) can occur at any level of organismal

hierarchy including biochemistry, physiology, morphology, and behavior (Pigliucci,

2001; Schlichting and Smith, 2002; Sih, 2004). The timescale of the plastic response

varies from acute (Brenowitz et al., 1998; Sih, 2004) to broadly ontogenetic (West-

Eberhard, 2003). Adaptive evolution of a plastic phenotype requires genetic variation for the plastic response or genotype by environment (G X E) interactions in the production of phenotypic variation. Plasticity and G x E interactions are well studied (Via and Lande, 1985; Stearns, 1989; West-Eberhard, 2003), with hundreds of studies finding variation in plasticity on which selection can act (Newman, 1992; Pigliucci, 2001; Dewitt and Scheiner, 2004a).

Variation in environment over the ontogeny of an organism creates numerous inputs into trait variation. Developmental plasticity, in which juvenile environment molds adult phenotype, is common (West-Eberhard, 2003) and may be beneficial if juvenile environment predicts adult environment. Shorter time-scale plastic effects, such as physiological acclimatization or context-sensitive behavior, are ubiquitous (Pigliucci, 2001; Sih, 2004) and likely to be more useful when the animal experiences unpredictable changes throughout its ontogeny. Although the combined influence of environmental effects across these scales has been largely ignored, it has recently been argued that current optimality models may fail as a consequence of conflicts between plasticity at different scales (Sih, 2004).

Environmental effects on traits occurring at different time scales may be purely additive, or acute plasticity may be contingent on the developmental environment. Analogous to genetic factors, environments experienced sequentially during an animal's ontogeny combine to influence a phenotype, and their effects can be decomposed into additive and interaction sources of variation. Environmental interactions are a potentially critical and understudied source of phenotypic variation. Figure 1 provides a set of hypothetical phenotypic effects that result from ontogenetically distinct environments and

their interactions. It is presented in a typical format for genotype and environment effects (Via and Lande, 1987), but in this case, different reaction norms represent differing developmental environments rather than differing genotypes. Interactions of the type in Figure 1C should be represented by environment by environment (E X E) interactions in a traditional quantitative genetic framework. Explicit measures of this type of environmental interaction in the production of phenotype are unexplored in quantitative genetics.

In this study, we differentiate between the developmental environment and the acute behavioral environment as causes of aggression and avoidance behavior in Zebrafish. We tested for plastic response by rearing laboratory populations of Zebrafish from egg to adulthood in normoxic and hypoxic conditions. We then measured aggression and avoidance levels in fish from both developmental treatments in both environments. We further tested whether plastic responses declined during a period of physiological adjustment (acclimation).

CHAPTER II

MATERIALS AND METHODS

We chose Zebrafish as a focal species and oxygen concentration as our

environmental variable for the following reasons. Hypoxia presents a common stress in

many fish species, producing a suite of organismal, cellular, and genetic responses

(Gracy et al., 2001; Nikinmaa, 2002; Ton et al., 2003). Hypoxic stress in fish can be

acute, seasonal, or chronic and hypoxia has been demonstrated to cause developmental

delays (Rombough, 1988) and alter the regulation of over 100 genes (Gracy et al., 2001;

 Ton et al., 2003) in several fish species. Plastic response of fish to hypoxia stress has

been demonstrated to produce ecologically relevant morphological tradeoffs (Chapman et

al., 2000). Finally, because Zebrafish are found across a wide variety of habitats from

fast-moving rivers to slow moving stagnant bodies of water (Talwar and Jhingran, 1991),

they are a good candidate to display complex plastic behavior in response to oxygen

level.

Stocks and Developmental Conditions

 Eggs used in the experiment were the product of individual mating of parents

from three different sources: wild-caught fish from India, commercially bred fish with a

history of regular backcrossing to wild fish, and relatively inbred commercial laboratory

strains (trade name ''Longfin Gold''). Wild stocks were obtained through Poseidon

Aquatics (Gardena, CA), and both commercial strains were obtained from

Scientific Hatcheries Inc. (Huntington Beach, CA). An assortment of crosses between

and within the available stocks produced 631 eggs from 12 full-sib clutches. Each clutch

was split with an equal number of eggs placed in each environment. For logistical

reasons all fish within a developmental environment were pooled as the individual fish

matured (40 days). Genetic variation could therefore not be partitioned within

environments. The genetic variation present was, however, balanced across the two

developmental environments and randomized across behavioral environments. This

allowed testing for the average plastic response across a diversity of genotypes. The

average survivorship to day 40 across clutches was not significantly different between

oxygen treatments (paired

t-test $P = 0.390$) with normoxic and hypoxic survivorship being 69% and 64%,

respectively.

Within two hours post-fertilization, eggs were placed in their developmental

environment. A split sump system of 20 tanks (total volume approx. 1200 l) allowed

passive and active exchange of water between the normoxic and hypoxic tanks.

Normoxic tanks were fed with water that was continuously oxygenated to near saturation

(DO approx. 6.8 mg/l) using airstone bubblers on the normoxic side of the sump. The

hypoxic tank's DO concentration was maintained at 0.8 mg/l (+/- 0.1 mg/l) by sparging

with N2 in a series of 2 m tall sparging columns controlled by a YSI 5200 monitoring

system. Light was provided on a 14L : 10D cycle using built in control systems in the

rearing rooms. A push-pull heating and chilling system maintained the temperature at 27

+/- 0.5 C. All fish were fed twice daily on a commercial diet obtained from Scientific

Hatcheries Inc. The fish were raised in those conditions to adulthood (roughly 75 days)

when they were assayed.

Experimental Designs

Two aggression experiments were carried out using the split clutches. In the first

of these experiments (no acclimation experiment), 128 fish from each developmental

environment were assayed in two complete blocks (64 fish per block). Each fish was

taken from its developmental environment and placed in an environmental chamber that

contained either hypoxic or normoxic water. The fish was then given a 10-min

behavioral adjustment (accommodation) period in a test arena. After accommodation, the

fish's behavior was videotaped for two min in order to determine the aggressiveness of

the fish. Half of the fish were tested in their own developmental environment and the

other half were tested in the opposite environment (i.e., there were no repeated

measures).

The second experiment (16-h acclimation experiment) was identical to the first

except that a new sample of fish from each developmental environment was used (one

block of 64 fish) and the fish were placed into a holding tank in the oxygen environment

that they would be assayed in for 16 h prior to being placed into the behavioral chamber.

This allowed animals to acclimate to the oxygen environment that they would experience

in the assay. After this acclimation period, they were moved into the assay chamber and

given a 10-min accommodation period before being videotaped for two min as in the first

experiment.

Behavioral Assays

The aggression assays were performed in 30 x 30 x 30 cm open top opaque white

polyethylene tank. The opacity of the material helped ensure that the fish were secluded

from outside disturbances. The tank was filled to roughly 20 cm depth and supplied with

a continuous flow of water from the appropriate sump for their treatment. The flow

through rate was adjusted so that there was no visible effect of currents on fish swimming

in the immediate vicinity (approx. 1 cm) of the inflow or drain, allowing the fish to swim

about the tank freely. Dissolved oxygen for the behavioral environments were not

detectably different from the equivalent rearing environment and shared the same

chemical composition and temperature as the rearing environments.

A mirror image of the subject provided a stimulus for aggressive behavior.

Mirror image stimulation (MIS) is a well-established method for studying fish behavior

(Tinbergen, 1951). Mirror image stimulation has the unique benefit of providing

 immediate feedback to the subject's activity and alleviates the need for invasive

procedures and excess handling (Rowland, 1999). A 10.16 cm high by 7.6 cm wide

stainless steel mirror was suspended below the waterline against the center of one wall of

the behavioral chamber. The time spent (in seconds) butting or nipping the mirror was

recorded as aggressive behavior when the tapes were reviewed. In normal stocking

densities, we have not noticed any schooling behavior of Zebrafish, while chases and fin

nipping are common. In pilot two-way behavioral experiments (West, unpubl. data),

such direct contact also always resulted in either fin nipping or forceful collisions. Given

that these interactions were likely to cause damage to one or both fish, we felt confident

that direct contact of a fish with its mirror image could conservatively be construed as

aggressive behavior rather than a schooling response.

A plastic aquarium plant was placed near the opposite end of the tank from the

mirror in order to provide a hiding place in which fish could take refuge from their own reflection. The time (in seconds) spent hiding in the foliage was used as a measure of avoidance behavior. We use the term avoidance in general and not specifically as an avoidance of the mirror image. Fish were only counted as hiding if they pushed into the foliage (i.e., utilized the cover) and remained stationary in that cover. General preference for the vegetation, when fish frequented the cover but did not utilize it, was not recorded as avoidance. Immediately after each assay was complete, the wet weight of that fish was recorded.

Statistical Methods

Developmental environment, behavioral environment (i.e., acute test environment), and their interaction were each of interest as possible sources of variance in both experiments. An ANCOVA model was used to test for developmental environment (E_{DEV}), behavioral environment (E_{BEHAV}), and an E_{DEV} by E_{BEHAV} interaction (E_{DEV} x E_{BEHAV}) as sources of variation in aggression and avoidance behavior. The across-treatment influence of wet weight was statistically removed since fish size might have influenced levels of aggression. Analyses were performed as ANCOVAs in the SAS GLM procedure (SAS Institute Inc., Cary, NC) with wet weight treated as a covariate. A t-test was performed to test for differences between the developmental treatments in wet weight. Block was included as a source of variation in the analysis of the no acclimation experiment while the 16-h acclimation experiment was completed in a single block. Since the average time spent in aggression and hiding is relatively low, a Poisson rather than normal distribution of residuals was expected in the data (Sneedecor

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and Cochran, 1989). We therefore applied square root transformations to both response variables to improve the normality of the residuals.

CHAPTER III

RESULTS

No Acclimation Experiment

E_{DEV} was a significant source of variation in aggression (Table 1a) and avoidance

(Table 2a). The average aggression of hypoxia-reared fish was lower than that of

 normoxia-reared fish (Fig. 2a), and hypoxia-reared fish spent more time in hiding than

did normoxia-reared fish (Fig. 3a). E_{BEHAV} did not contribute to the variation in

aggression except through a significant E_{DEV} x E_{BEHAV} interaction. Fish were, on

average, more aggressive in their own developmental environment (Fig. 2a). E_{BEHAV} did

not affect avoidance behavior (Fig. 3a) either directly or through an E_{DEV} x E_{BEHAV}

interaction (Table 2a).

Wet weight added significantly to the variation in aggression (Table 1a) with

larger fish being more aggressive. Wet weight was also a significant source of variation

 in avoidance behavior (Table 2a), with larger fish spending less time on avoidance. A t-

test showed no significant difference in wet weight between the two developmental

environments for this experiment ($P > 0.773$).

16-h Acclimation Experiment

Neither E_{DEV} nor E_{BEHAV} were significant sources of variation in aggression

except through an E_{DEV} X E_{BEHAV} interaction (Table 1b). In hypoxia, hypoxia-reared fish

were most aggressive, while in normoxia, normoxia-reared fish were most aggressive (Fig. 2b). Only E$_{DEV}$ was a significant source of variation in avoidance behavior (Table 2b). Across both environments, hypoxia reared fish again spent more time hiding in the vegetation (Fig. 3b).

Wet weight was not a significant source of variation in aggression (Table 1b) or avoidance behavior (Table 2b). A t-test showed no significant difference in wet weight between the two developmental environments for this experiment ($P > 0.09$). We ran the model both with and without wet weight as a covariate and again found no qualitative changes resulting from the inclusion of wet weight.

CHAPTER IV

DISCUSSION

Hypoxic stress during development clearly affects the behavioral response of

Zebrafish. However, the response is not simply an increase or reduction in aggressive

behavior. Instead, the effect of developmental environment on aggression is conditional

on the behavioral environment (Table 1). Fish are most aggressive in their native

environment (Fig. 2). In Zebrafish, which experience a broad range of environments,

 (Talwar and Jhingran, 1991), this may affect the ecological and evolutionary dynamics

of the species.

It is likely that aggressive behavior is energetically costly for a fish and this cost

may be exacerbated when fish are moved to a novel oxygen environment. In this

experiment, normoxia-reared fish were just as aggressive as hypoxia-reared fish when

exposed to the hypoxia for less than 15 min (Fig. 2A). However, when they were

acclimated to hypoxia for 16 h, they did not maintain that high level of aggression and

were less aggressive than hypoxia-reared fish (Fig. 2B). This pattern suggests that while

in this case developmental plasticity may be adaptive, behavioral plasticity may be

 partially due to nonadaptive constraints. It appears that in hypoxic water, normoxia-

reared fish may be incapable of maintaining costly behaviors and vice versa. Zebrafish

reared in normoxia and placed in hypoxic water produce significantly higher lactic acid

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concentrations compared to hypoxia-reared Zebrafish (Widmer, Moore, and Bagatto, unpubl. data) indicating that oxygen may limit behavioral dynamics. The pattern of environmentally contingent behavior we found is in keeping with a correlated behavioral response to developmental changes in physiological or morphological traits that confer an advantage in a constant habitat.

Avoidance behavior in the presence of a mirror image stimulus was not contingent on the behavioral environment (Table 2). Across both behavioral environments, hypoxia-reared fish spent more time hiding than normoxia-reared fish and neither group of fish substantially altered their hiding behavior in response to behavioral environment. In short, for avoidance there was no behavioral plasticity. Developmental plasticity leading to avoidance behavior in hypoxia-reared fish could be either an adaptive ''energy saving'' strategy or a non-adaptive consequence of a lack of available resources for more energetically costly pursuits.

The adaptive benefit of plasticity of aggression and avoidance behaviors is not fully resolvable within this study. There is, however, ample reason to think that such behavior may influence the dynamics of Zebrafish populations. Developmentally plastic organisms expressing unique phenotypes under the influence of a novel environment could allow invasion of that habitat, facilitating further adaptation. Ontogenetic factors clearly influence behaviors that may be critical in determining the success of Zebrafish entering novel oxygen regimes. These changes result in fish that are more timid in novel oxygen environments. This could alter the rates of adaptation to a changing habitat, range expansion or contraction, and competition as well as other evolutionary and ecological processes. Convincing arguments have been made for plasticity as an agent of

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micro- and macroevolutionary change in general (Via and Lande, 1985; Agrawal, 2001; West-Eberhard, 2003). Plastic responses have been recognized as being necessary for colonization of novel habitats (West-Eberhard, 1989; Agrawal, 2001), and plasticity in this case could allow invasion of novel habitats if lowered aggression is optimal in those habitats. This particular pattern of plastic response could also be an impediment to invasion of, and adaptation to, a novel environment if aggressive behavior is lowered below its optimum level in that novel environment.

We found that $E_{DEV} \times E_{BEHAV}$ interactions are a critical source of variation in aggressive behavior in Zebrafish. The interaction between the developmental and adult environments is relatively poorly understood from an evolutionary perspective. There are, nevertheless, certain areas in which these types of interactions may be common. Specifically, learned and conditioned responses represent a case where varied experience earlier in life dictates the behavior of individuals later in life (Sih, 2004). For instance, in the squid, *Loligo opalescens*, exposure to copepods during early development determines prey capture behavior during later developmental stages (Chen et al., 1996). In such cases, prediction of an individual's behavior requires knowledge of both the current environment and previous environments.

In this study, we have looked at the interaction between the developmental environment and the adult environment in the creation of behavioral phenotypes. We introduce E X E interactions as a novel means of quantifying the integrated influence of ontogenetic environments. Sih (2004) has recently pointed out the potential importance of such joint developmental and behavioral responses in shaping evolutionary change, although in a different heuristic context. In general, when E X E interactions affect

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phenotypic variation in any type of trait, we must account for those interactions if we are to understand the evolutionary dynamics of those traits.

In summary, Zebrafish display plasticity in both aggression and avoidance across oxygen environments. In the case of aggression, it is the interaction of developmental environment and the behavioral environment that determines a fish's responses. In the case of avoidance, only the developmental environment influences the behavior. The existence of complex plasticity in aggressive behavior across oxygen environments indicates that more study of the adaptive significance of this particular trait is necessary. We conclude that a simple expansion of current studies to detect E X E interactions may elucidate the extent to which multiple environments seen during ontogeny combine to determine phenotype.

Table 1: Aggression analysis.   Analysis of covariance for time spent on aggressive behavior (A) without and (B) with acclimation. Sources of variation included developmental environment (E_{DEV}), behavioral environment (E_{BEHAV}), the interaction between developmental and behavioral environments (E_{DEV} X E_{BEHAV}) and block. Wet weight was included in the analysis as a continuous covariate.

Source	DF	M S	F	P
Wet weight	1	34.765	6.84	0.0100
A) Without Acclimation				
Block	1	41.321	8.13	0.0051
E_{DEV}	1	42.220	8.31	0.0047
E_{BEHAV}	1	0.432	0.09	0.7711
E_{DEV} X E_{BEHAV}	1	34.237	6.74	0.0106
Error	122	5.080		
B) With Acclimation				
Wet weight	1	0.399	0.100	0.7566
E_{DEV}	1	< 0.000	< 0.000	0.9996
E_{BEHAV}	1	5.956	1.450	0.2337
E_{DEV} X E_{BEHAV}	1	29.290	7.120	0.0098
Error	59	4.115		

Table 2: Avoidance analysis. Analysis of covariance for time spent on avoidance behavior (A) without and (B) with acclimation. Sources of variation included developmental environment (E_{DEV}), behavioral environment (E_{BEHAV}), the interaction between developmental and behavioral environments (E_{DEV} X E_{BEHAV}) and Block. Wet weight was included in the analysis as a continuous covariate.

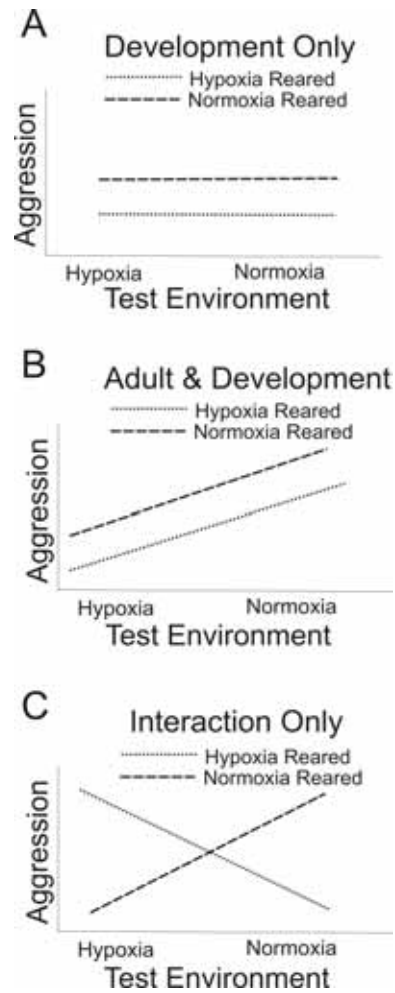| Source	DF	M S	F	P
Wet weight	1	23.647	4.97	0.0276
A) Without Acclimation				
Block	1	2.647	0.56	0.4570
E_{DEV}	1	58.985	12.40	0.0006
E_{BEHAV}	1	4.612	0.97	0.3266
E_{DEV} X E_{BEHAV}	1	1.395	0.41	0.5247
Error	122	4.755		
B) With Acclimation				
Wet weight	1	6.883	0.87	0.3588
E_{DEV}	1	39.544	4.98	0.0295
E_{BEHAV}	1	7.167	0.90	0.3461
E_{DEV} X E_{BEHAV}	1	1.107	0.14	0.7102
Error	59	7.946		

Figure 1: Hypothetical norms of reaction representing plastic response to (A) developmental environment only, (B) acute and developmental environment acting additively, and (C) an interaction where developmental environment influences aggression only by reversing the effect of acute environment or vice versa. The solid line represents the response of normoxia-reared individuals across environments. The dashed line represents the response of hypoxia-reared individuals across environments.
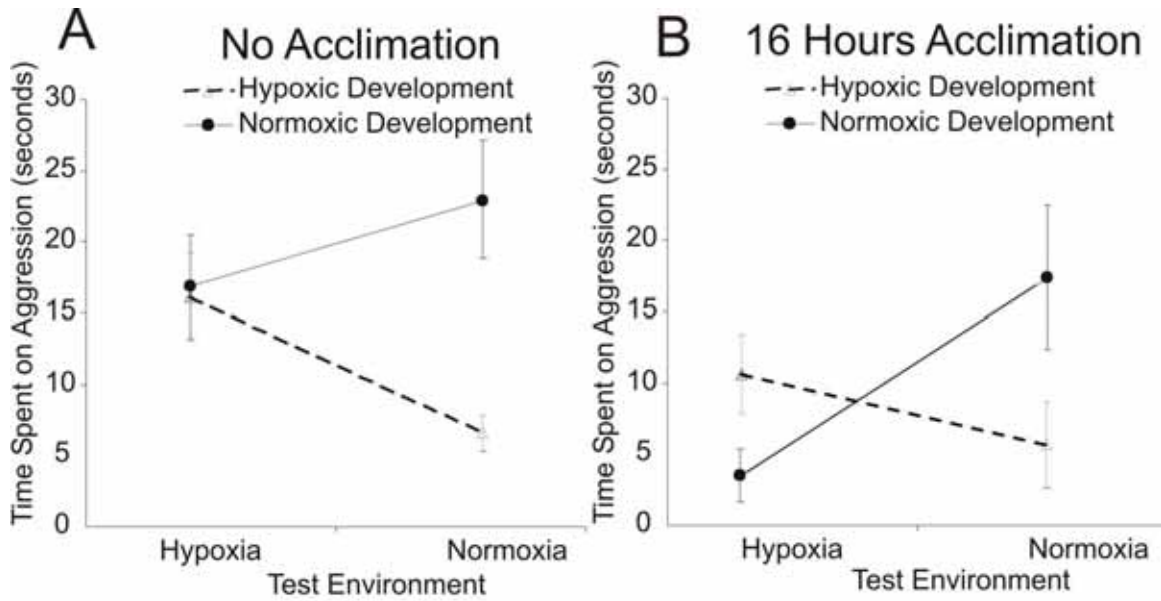
Figure 2: Aggression results. The influence of environment on aggression (A) without acclimation to the environment and (B) with 16 h of acclimation. Open and solid symbols represent means for hypoxic- and normoxic- reared fish, respectively. Error bars represent standard errors.
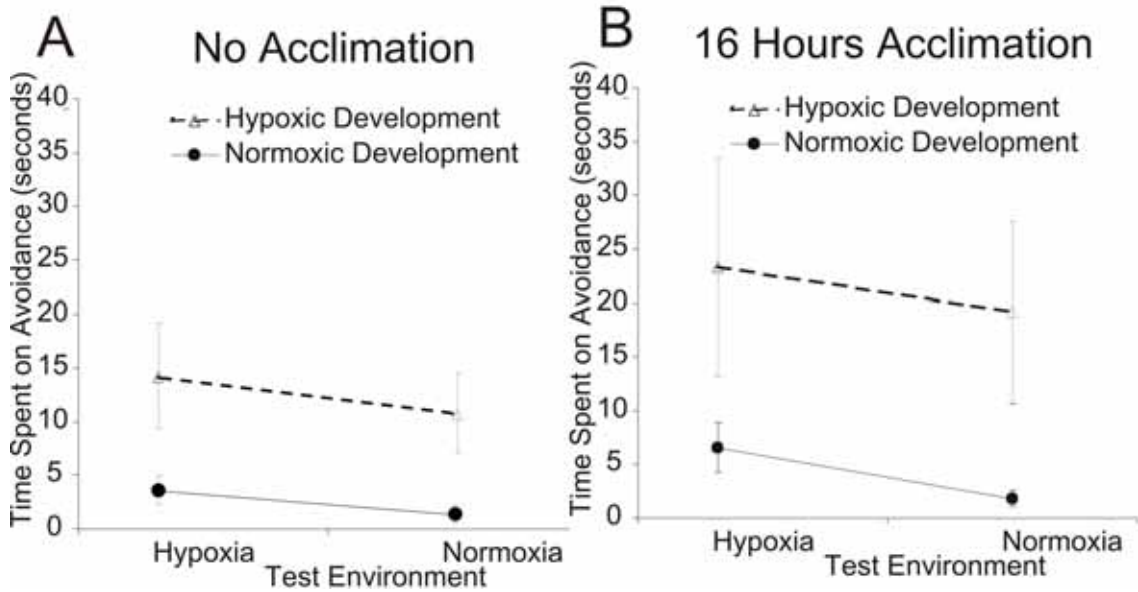

Figure 3: Avoidance results. The influence of environment on avoidance (A) without acclimation to the environment and (B) with 16 h of acclimation. Open and solid symbols represent means for hypoxic- and normoxia-reared fish, respectively. Error bars represent standard errors.

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