Swordtails Gone Wild: How Maternal Environment, the Aquarium Trade, and Artificial Selection Influence Behavior and Invasive Potential in a Popular Pet Fish

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ABSTRACT

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Swordtails Gone Wild: How Maternal Environment, the Aquarium Trade, and Artificial Selection Influence Behavior and Invasive Potential in a Popular Pet Fish

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While non-native species vary greatly in their ability to succeed as invasive species, the mechanisms leading to successful invasions are not well understood, especially for intentional introductions. Understanding the extent to which these differences are due aspects of the invasive process could lead to better mitigation of invasive species. In this dissertation, I investigated the effect of the invasive process acting as *a filter,* on behavior, behavioral variability, and behavioral syndromes in ways that could explain the invasive success of the *Xiphophorus helleri-maculatus* hybrid, an intentionally introduced species. I measured three behaviors (boldness, exploration, and aggression), and while boldness and aggression increased throughout this process, I did not find an overall filtering effect leading to an invasive syndrome. To dive deeper into the possible causes of changes in these three behaviors, I also investigated the roles played by different color morphology, and whether different breeding practices (e.g., selective breeding, free mate choice) could influence behaviors. By comparing the same three behaviors across three color morphs of swordtail hybrids with varying degrees of melanin, I found differences in behavior and female fecundity. While the most melanic fishes expressed higher levels of boldness, aggression, and exploration, it also had the lowest fecundity, suggesting that it likely did not have more 'invasive potential' than the

other morphs. To better understand if these changes were related to melanin or the breeding practices used to produce this extreme coloration, I compared other fish of various colors produced via selective breeding to a population of fish that did not undergo this rigorous form of artificial selection. Contrary to my expectations, I did not find a difference in behavior between fishes produced by different breeding practices. While females that underwent an additional stage of selective breeding produced more offspring, this was likely due to systematic overfeeding of fish in breeding pools right before sale, and not a result of mechanism of the selective breeding process. Ultimately, the invasive process and some aspects of the pet trade, such as selective breeding and overfeeding, have influenced the behaviors and reproductive output of *X. helleri-maculatus* hybrids. This research corroborates similar findings on the change in behaviors across stages of invasion in unintentional introductions. Notably, my findings also suggests that intentional introductions of animals that underwent selective pressures in the pet trade may show an increase in invasive potential, especially if introduced populations are continuously augmented with different types of individuals (e.g., color morphs, personalities).

DEDICATION

To my husband, for standing by me in the good times and the bad, for chasing swordtails through beautiful streams and disastrous creeks, for cheering my successes and mourning my failures alongside me. I would not have made it this far without you.

; and so she goes on

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The care and study of these animals was approved by the Institutional Animal Care and Use Committee of Ohio University (IACUC number 12-L-042).

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This chapter has been accepted as: Maternal investment influences development of behavioural syndrome in swordtail fish, *Xiphophorus multilineatus* in the journal *Animal Behaviour*, published by Elsevier Ltd. The formal publication is available at: dx.doi.org/10.1016/j.anbehav.2015.02.013

Abstract

Despite a rising interest in behavioral syndromes (correlations between different behaviors across context and time), the development of these syndromes is not well understood. Using the swordtail fish *Xiphophorus multilineatus*, we looked at the effects of maternal investment and social environment during development on the formation of a behavioral syndrome. A previous study demonstrated that the diet on which mothers were reared, controlling for their size, influenced the growth rate and size of their sons: females reared on high-quality diets had sons that grew faster and were larger at sexual maturity. In addition, males that were exposed to an adult male during development grew faster. We examined the behaviors of these same males across two contexts: aggression towards a mirror image and boldness under threat of predation. We detected an influence of maternal investment but not of social environment on the formation of a behavioral syndrome. The sons of mothers reared on high quality diets exhibited a positive correlation between boldness and aggression, while there was no correlation among the sons of mothers reared on low-quality diets. Maternal investment influenced the formation of the syndrome by producing changes in only one of the behaviors, the behavior most closely linked to energy acquisition (boldness under risk of predation) rather than energy expenditure (aggression). Our results suggest that energy balances could help predict the circumstances that would favor the formation of behavioral syndromes. Our results also support the hypothesis that behaviors involved in energy acquisition as compared to energy use will be more sensitive to fluctuations in variation in resources.

Introduction

Behavioral syndromes are consistent correlations of behaviors across contexts and time within a population (Sih et al. 2004), and understanding their development will not only provide valuable insights into the adaptive costs and benefits of behavioral plasticity, but are important for designing the studies to examine their causation and evolution (Stamps and Groothuis 2010). Behaviors that are not independent across varying situations can persist if the errors of individual behavioral types cost less than remaining behaviorally plastic, assuming the information required to behave optimally is available in a given context (Bell 2007, Huntingford 2004, Sih et al. 2004). However, if the development of syndromes is plastic, the benefits of correlated behaviors could be achieved in some environments without the costs associated with syndromes in other environments (Stamps 2007). Despite growing interest in behavioral syndromes across taxa, our understanding of their development is limited (Stamps and Groothuis 2010). In this study, we examined the influence of maternal investment and early social environment on the development of a behavioral syndrome in the swordtail fish, *Xiphophorus multilineatus*, to gain further insights into the factors influencing its development.

Mothers across taxa produce inherited environmental effects through a variety of mechanisms, such as adjusting the sex ratio within a brood, manipulating brood size, and adjusting hormonal and nutritional resources partitioned to individual offspring (Mousseau and Fox 1998). Variation in resource allocations to eggs influences metabolic and growth rates in brown trout, *Salmo trutta* (Regnier et al. 2010) and swordtails (*X. multilineatus*: Murphy et al. 2014, Rios-Cardenas et al. 2013), and growth rates have been suggested to be associated with the development of behavioral types and syndromes in fish (Huntingford 2004, Regnier et al. 2010). These maternal effects are not limited to fish. Variation in prenatal hormonal investment influences the responses of adult zebra finches, *Taeniopygia guttata*, towards novel stimuli (Tobler and Sandell 2007), the reproductive behavior of adult house sparrows, *Passer domesticus* (Partecke and Schwabl 2008), as well as growth rates and escape behaviors in garter snakes, *Thamnophis elegan*s (Robert et al. 2009). In addition, the juvenile social environment has been hypothesized to influence the development of a behavioral syndrome (Huntingford 2004) and has been shown to influence individual behaviors such as aggression (e.g. green swordtail, *Xiphophorus helleri*: Hannes & Franck 1983; mangrove killifish, *Kryptolebias marmoratus*: Edenbrow and Croft 2012) and social dominance (e.g. blue tits, *Cyanistes* *caeruleus*, and great tits, *Parus major*: Hansen and Slagsvold 2004) in adult animals. However, the extent to which maternal effects and social environment influence the development of behavioral syndromes has not been previously examined.

We tested the behaviors of males of the swordtail *X. multilineatus* to investigate the extent to which maternal investment and social environment influence correlations between behaviors across two contexts: aggression towards a mirror image and boldness under risk of predation. Murphy et al. (2014) found that both maternal investment and early social environment influenced growth rates in these males. Controlling for mother's size, mothers reared on high-quality diets produced fry that were larger, grew faster and were larger at sexual maturity (Murphy et al. 2014). In addition, male offspring exposed to an adult male during early development grew faster than control male offspring. Given the hypothesis that consistent differences in growth rates can lead to the development of behavioral syndromes (Huntingford et al. 2010, Stamps 2007), we hypothesized that variation in maternal investment and/or social experience as juveniles could influence the formation of a behavioral syndrome. One of the interesting differences between the two behaviors we examined is their relationship to an individual's energy balance. Aggression is often associated with energy expenditure, whereas boldness is often associated with energy acquisition (Biro and Stamps 2010). This is very likely to be the case in swordtails as well. Male aggression towards conspecifics is primarily a behavior that uses energy; in nature, males defend access to groups of feeding females (Morris et al. 1992). In contrast, boldness under risk of predation is a behavior associated with energy acquisition, as being bold around a predator increases time available to forage. Alterations in food intake are predicted to have stronger effects on energy balance than alterations in expenditure (Prentice 2005). Therefore, we examined whether variation in maternal investment and early rearing environment would influence the behaviors from the two contexts independently, to determine whether variation in these two factors influences changes in both behaviors. We predicted that the behavior involved in energy acquisition would be more sensitive to environmental changes.

Methods

The *X. multilineatus* males we tested (N=60) were reared to sexual maturity by Murphy et al. (2014) to examine the influences of maternal investment on growth rate. The mothers of the males we examined were laboratory reared to sexual maturity by Lyons et al. (2013) on two diets (high quality versus low quality) that differed primarily in protein content. The high-quality food treatment, Tetra-min Tropical Flakes (Tetra®, United Pet Group, Blacksburg, VA, U.S.A.), had 47% protein content and was supplemented by bloodworms three times a week; the low-quality food treatment, Nishikoi Wheat Germ Koi Food (Nishikoi®, Whitehall, Wethersfield, Essex, U.K.), had 20% protein content and was not supplemented. Protein content influences female reproductive performance in swordtail fish (Chong et al. 2004). Once females reached sexual maturity, they were all bred to males from the large size class (genotype Y-L), which controlled for differences in maternal allocation based on male alternative reproductive tactic (Rios-Cardenas et al. 2013). Offspring of each brood were individually isolated at 2 weeks of age in separate 5-gallon (18.9-litre) ‘home’ aquaria and maintained on the low-quality diet treatment. At 70 days of age, half of the fry in each brood were presented with an adult courter male in a fry box (a clear plastic box that allows water exchange without physical contact) once daily for 5 min, for 2 weeks; the other half of the fry were presented with an empty fry box for the same amount of time. Twenty-eight courter males measuring 32-47 mm standard length (SL) were used for the exposure treatment. Growth rates were measured by subtracting size (measured as SL) at 70 days (beginning of social treatment) from size at 130 days (prior to sexual maturity) and dividing by 60 days (see Murphy et al. 2014). We tested males for aggression (mirror test) and boldness (predator inspection test) 14-17 weeks after sexual maturity, with 24-28 h between the two tests. We tested fish for aggression prior to testing for boldness to decrease the possibility of carryover effects (Bell 2013) and because fish exposed to predators take longer to recover than fish exposed to a conspecific or mirror image (Bell et al. 2007).

Mirror Test (Aggression)

Fish were left in their individual tanks, their established ‘home territory’, for the aggression test. One hour before testing, we removed all refugia from the tank. Gravel substrate was left undisturbed. Then, the focal fish was presented with a mirror on the outside of its tank lengthwise and observed for 300 s. We recorded time until first approach (latency, scored as negative numbers, with lower numbers being more aggressive), number of displays, number of bites and number of approaches (following Moretz and Morris 2003). After testing, the mirror was removed and all refugia were replaced.

Predator Inspection Test (Boldness)

The predator *Herichthys cyanoguttatus*, which is similar to predators of *X. multilineatus*, was placed in one of two side compartments of the test tank (50-gallon (189.3-litre); each side compartment, 47 x 32 x 43 cm; central compartment, 73 x 32 x 43 cm), separated by glass. This allowed visual recognition of the predator without water exchange or physical contact. The focal fish was then placed in an opaque tube at the center of the test tank. After 60 s of acclimation, we released the fish by lifting the tube out of tank (via a string from behind a viewing screen). The focal fish was observed for 300 s. We recorded the time spent in the third of the tank closest to the predator, the time until first approach and the number of approaches (following Bell and Stamps 2004).

Analysis

We used a principal component analysis to determine which of the behaviors measured in each context best explained variation in behavior (i.e. heaviest loading and most consistent across treatments). We used latency to approach the mirror as our measure of aggression, and number of predator approaches as our measure of boldness (see Table 1.1). Determining whether the maternal diet and early social environment affected the behaviors in one or both contexts required the use of a different type of analysis than is typically used in studies of behavioral syndromes (i.e. we used separate analyses of the behaviors in each context). We examined how the two variables known to influence growth rates (mother's diet, social treatment as juvenile) influenced behaviors with two mixed models, one with each behavior as the dependent variable. The models included mother's diet and social treatment as independent variables, the mother's identity as a random factor and the behavior in the other context as a covariate. If variation of the behavior in the other context explains significant variation in the dependent variable (the other behavior), this suggests the two behaviors are correlated (form a behavioral syndrome).

Table 1.1

Loadings on the first principal component for the principal component analysis of boldness under risk of predation and aggression towards a mirror image in swordtail fish, *X. multilineatus*

	HQ	LQ
Boldness		
Time with predator	0.280	0.122
Movements	0.448	0.463
Areas used	0.581	0.555
Predators approaches	0.619	0.680
Aggression		
Latency to approach	0.670	0.634
Bites	0.721	0.540
Displays	-0.180	0.553

HQ: high-quality diet; LQ: low-quality diet.

The measure of aggression did not conform to the assumptions of a parametric model even after using a generalized model. Therefore, we used a linear mixed model on rank-transformed data. For the analysis of number of approaches to predator (measure of boldness), we used a generalized mixed model with a Poisson distribution and a log link function. We found the minimal adequate model for each, using a model simplification based on the Akaike's Information Criterion (AIC). Models were discarded based on the criteria of a difference of more than two Akaike points from the minimal model. Given the significant interaction between diet and aggression on variation in boldness (see Results), we also examined the correlation between aggression and boldness for the two subsets of males (sons of high-quality mothers and sons of low-quality mothers) with linear regressions, using a Bonferroni correction.

Ethical Note

Although mild, temporary physiological stress may have occurred during behavioral trials, we took a number of steps to minimize stress to the animals. In the case of offspring social experience and predator observations, focal fish were allowed to see but not interact with stimulus fish, preventing any physical contact and potential harm to either individual. Observations of aggression and boldness were kept short (5 min) and tanks were treated with 5 ml of Stress Coat® (Aquarium Pharmaceuticals API, Chalfont, PA, U.S.A.) immediately after each trial to reduce the release of stress-mediated hormones into the water. Stress Coat® has been shown to decrease water cortisol levels by 40% 7 h after handling (common goldfish, *Crassus auratus*: Snellgrove et al., n.d.). Animals on the low-quality diet did not have higher mortality (Morris, n.d.) or any indicators of malnutrition. The care and study of these animals was approved by the Institutional Animal Care and Use Committee of Ohio University (IACUC number 12-L-042).

Results

An aggressive-bold/submissive-shy behavioral syndrome was detected, with mother's diet influencing the strength of this relationship. In the analyses of variation of both behaviors, the behavior in the other context had a significant influence: variation in aggression had a significant influence on variation in boldness, and variation in boldness had a significant influence on variation in aggression (Table 1.2).

Table 1.2

Variables influencing boldness under risk of predation in swordtail fish, *X. multilineatus* (generalized mixed model) and aggression towards mirror image (linear mixed model)

Variables	F	df	p
Boldness			
Diet	10.309	1, 58	**0.002**
Aggression	4.542	1, 58	**0.037**
Aggression			
Diet	2.739	1, 55	0.104
Treatment	0.395	1, 55	0.532
Boldness	4.937	1, 55	**0.030**
Diet x Boldness	5.918	1, 55	**0.018**
Diet x Treatment	0.012	1, 55	0.914

Significant P values are shown in bold.Diet: mother's diet (high quality versus low quality); Treatment: social treatment during development (exposed to adult male versus empty box).

Maternal investment (mother's diet) had a significant influence on boldness but not on aggression. Sons of mothers reared on the high-quality diet were overall less bold (estimated mean boldness score: 1.444) than sons of mothers reared on the low-quality diet (estimated mean boldness score: 2.360) ($P < 0.05$; Table 2). Maternal investment only influenced aggression through an interaction with variation in boldness (Table 1.2).

The correlation between aggression and boldness was statistically significant for sons of mothers reared on the high-quality diet ($R^2 = 0.277$, $F_{1,23} = 8.812$, $P = 0.007$, $\alpha' = 0.025$) but not for sons of mothers reared on the low-quality diet ($R^2 = 0.001$, $F_{1,34} = 0.035$, $P = 0.852$; Figure 1.1).

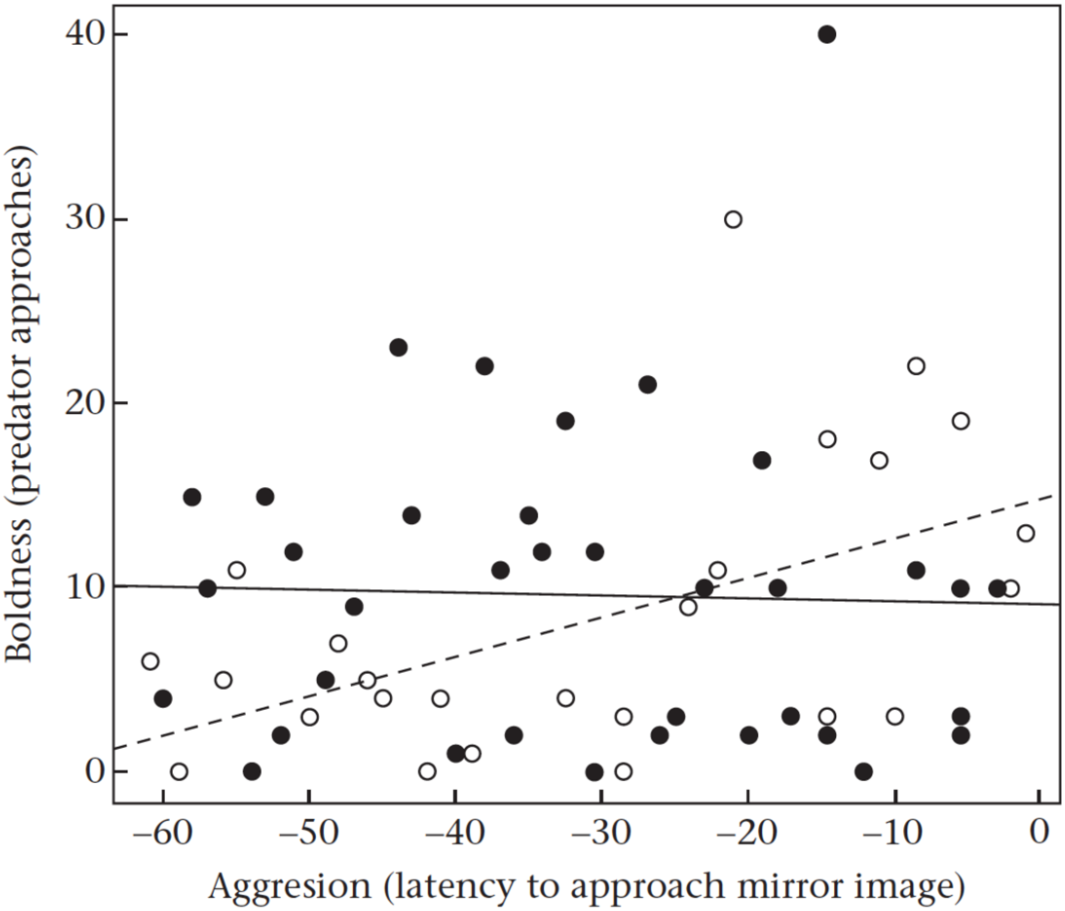


Figure 1.1 Relationship between aggression and boldness for sons of mothers reared on high-quality diets (open circles, dashed line) and low-quality diets (closed circles, solid line). Aggression (latency to approach, 0 = attacked most quickly, i.e. more aggressive) and boldness (number of predator approaches) were rank transformed for normality.

Social treatment was included in the best model for the analysis of aggression, but had no significant effects on either behavior.

Discussion

Understanding how behavioral syndromes form will help us understand why they form. We detected an influence of maternal investment (inherited environmental effects) on the development of a behavioral syndrome in the swordtail fish, *X. multilineatus*. The classic syndrome between boldness under risk and aggression towards a mirror image was plastic, developing across the sons of mothers reared on a high-quality diet (hereafter HQ mothers), but not across the sons of mothers reared on a low-quality diet (LQ mothers). The difference was due to lower boldness scores for the less aggressive sons of HQ mothers as compared to the less aggressive sons of LQ mothers. We suggest that the presence or absence of the behavioral syndrome is best explained in the context of trade-offs between energy acquisition and use (Stamps 2007, 'energy balance' hypothesis), with maternal investment influencing the formation of the correlation by increasing individual's initial energy reserves and/or growth rates.

The influences of maternal investment on males detected by Murphy et al. (2014) can provide insights into why we detected a behavioral syndrome in sons of HQ mothers, but not in sons of LQ mothers. Sons of HQ mothers grew faster and were larger at sexual maturity (Murphy et al. 2014). Thus, larger size itself could directly explain the reduction in risky behaviors (boldness) of the males with increased maternal investment. However, given that larger male swordtails are less susceptible to predation than smaller males (Basolo and Wagner 2004), larger males might be predicted to increase boldness, if the development of the syndrome were based on body size alone. Instead, we think that an 'energy balance' hypothesis (modified from the 'energy budget' hypothesis, Biro and

Stamps 2010) better explains the development of the behavioral syndrome we detected. Males that received more maternal investment could be less bold (resulting in lower feeding rates) due to their better start in life. Sons of LQ mothers might need to continue to be active and feed even in the presence of predators (increased boldness) to compensate for their poorer start in life. The energy balance hypothesis suggests that feeding rates that develop during periods of growth are maintained into adulthood, when male swordtails do not grow. In addition, it suggests that in a natural environment with predators, males with greater maternal investment would have yet another advantage: higher survival rates due to the use of less risky behaviors. However, there still needs to be an explanation for why the more aggressive, high maternal investment sons were bolder than the less aggressive, high maternal investment sons. From an ultimate perspective, the energy balance hypothesis for the formation of behavioral syndromes would predict that more aggressive (increased energy expenditure) individuals are under stronger selection for energy acquisition (increased boldness). An alternative hypothesis, which takes a mechanistic perspective and is therefore not necessarily mutually exclusive, is that it could be easier to produce a less aggressive individual that is bolder under risk of predation (sons of LQ mothers) than to decouple high levels of aggression from high risk taking behaviors such as boldness.

Boldness under risk of predation (energy acquisition) was more sensitive to maternal investment than was aggression (energy expenditure). This result supports the hypothesis that energy acquisition has more of an impact on energy balance than on energy use (Prentice 2005), as mothers' investment in their sons influenced the development of a behavior that would influence energy acquisition (boldness), but not one involved in energy use (aggression). Note, however, that these results may not apply to free-living fish, as our laboratory-reared fish had access to many more calories than do free-living fish and they were not dealing with predation or defense of females, which requires constant chasing of males off of the male's territory. Most of the well validated monogenic correlates with human obesity syndromes have effects primarily mediated through the regulation of appetite (i.e. acquisition of energy), rather than energy expenditure (Farooqi and O'Rahilly 2005, but see Cole et al. 2010). Variation in the copy number and alleles of melancortin-4 receptor gene (*MC4R*) is associated with age and size at sexual maturity for *X. multilineatus* (Lampert et al. 2010), and variation in this gene is associated with Type 2 diabetes (T2DM) in humans (Tao and Segaloff 2003, Ste Marie et al. 2000). Further study of variation in *MC4R* and the behaviors of swordtails across contexts that function in both energy acquisition and expenditure could improve our understanding of the role that this gene plays in obesity and T2DM in humans.

Growth rates are associated with a higher resting metabolic rate, which is strongly correlated with behaviors that influence an individual's food intake, such as boldness under risk of predation (Biro and Stamps 2010). In common carp, *Cyprinus carpio*, higher risk taking (boldness) and competitive ability (aggression) are associated with higher resting metabolic rates, resulting in a high risk-taking phenotype in the laboratory (Huntingford et al. 2010). A mechanism by which maternal investment could influence metabolic rate, and thus growth and behaviors involved in the syndrome we examined, is through variation in hormone allocation. Variation in egg hormone allocation (i.e.

cortisol, testosterone) is known to affect offspring's aggression, resting metabolic rate and growth rate across taxa depending on concentration and time of hormone introduction (Burton et al. 2011, Li et al. 2010, Sloman, 2010). Further studies are needed to determine whether the previously detected associations between growth rate and development of behavioral types (Regnier et al. 2010) and syndromes (Huntingford et al. 2010) could explain how maternal investment influences the development of behavioral syndromes in *X. multilineatus*. Hormone levels of offspring should be measured immediately after birth to better understand the potential differences between offspring of mothers on high- and low-quality diets. We also suggest expanding on the traits examined to include others that pertain to growth and metabolism, such as measures of activity or exploration.

We detected no effect of early social exposure on aggression or boldness, or on the relationship between the two behaviors, even though socially isolated swordtails have been shown to be more aggressive (Hannes and Franck 1983). One explanation for why we did not detect an influence of social exposure on aggression may be because our measure differed from that used by Hannes and Franck (1983). However, Hannes and Franck (1983) also reported that prolonged isolation (over 4 weeks) resulted in a decrease in aggression due to a possible lack of stimuli. In our present study, fish were isolated from 2 weeks of age until 70 days of age, when half were exposed to courter males (social treatment), and then all fish remained isolated until they were tested as adults (at 14-17 weeks of age). Therefore, it is possible that the lack of stimuli prior to testing reduced aggression for both groups. Future studies should expand behavioral assays to assess the influence of the timing and length of social exposure on aggression and the development of behavioral syndromes.

In conclusion, the development of a behavioral syndrome in the swordtail fish, *X. multilineatus*, was influenced by maternal investment, but not social exposure during juvenile development. These results suggest a potential relationship between growth, metabolic rates and an individual's behavioral type. In addition, the syndrome formed as a result of changes in only one of the two behaviors, the behavior most closely associated with energy acquisition. Our understanding of the adaptive benefits and evolutionary constraints of behavioral syndromes will improve with additional research on their development, as well as the extent to which energy balance influences the relationship between behaviors in different contexts.

CHAPTER 2: INVASIVE PROCESS DOES NOT PRODUCE ‘INVASIVE SYNDROME’ IN *XIPHOPHORUS HELLERI-MACULATUS*

Abstract

While it has been established that exotic species vary greatly in their ability to succeed as invasive species, what causes this extreme variability in success largely remains unknown. Understanding the extent to which these differences are due aspects of the invasive process could lead to better mitigation of invasive species. I investigated the effect of the invasive process acting as *a filter*, on behavior, behavioral variability, and behavioral syndromes in ways that could explain the invasive success of the *Xiphophorus helleri-maculatus* hybrid, an intentionally introduced species. I measured three behaviors (boldness, exploration, and aggression) known to play a role in invasive success across three populations representing different stages of invasion: fish from commercial breeding pools, fish found in pet stores, and fish in an established invasive population in Hawaii. I did find a trend indicating the increase of boldness, aggression, and exploration scores across populations. The invasive fish were bolder than those found in the pet trade, while fish from the pet trade were more aggressive than those in the breeding pools. Behavioral variance was not significantly different between breeding pool and pet trade fish, but was significantly lower in the invasive population when compared to the pet trade population. The relationships between the three behaviors were not significantly different across all populations, indicating this is not an invasive syndrome, as it was not

produced or changed by the invasive process. My results suggest that changes in individual behaviors and increases in behavioral variation caused by either hybridization or the invasive process may play more of a role in the success of *X. helleri-maculatus* as an invasive species than the formation of an invasive syndrome.

Introduction

Invasive species are a massive threat to biodiversity and the health of ecosystems worldwide. The behavior of invasive species has been identified as a potentially important factor of invasive success (Chapple et al. 2012, Holoway and Suarez 1999) but empirical studies often only examine single behaviors in the context of one stage of invasion. In most cases, this is the final stage (spread), when the exotic species is already well established (Chapple et al. 2012). However, despite numerous introductions, some species never become invasive, while others establish viable populations and begin to spread almost immediately. This is true for intentionally introduced species as well (Johnston and Purkis 2015). Some of this variation in success is likely due to propagule pressure (Blackburn et al. 2015, Johnston and Purkis 2015), and important life history traits (e.g. mortality, age to sexual maturity, see Johnston and Purkis 2015). Behavior may contribute directly to determining propagule pressure as well as other predictive characteristics of invasive success as it determines how individuals interact with their biotic and abiotic environment. Considering behavioral traits while investigating invasive biology has improved the understanding of, and the ability to predict, invasive species success (Chapple and Wong 2016, Carere and Gherardi 2013, Blackburn et al. 2009). A better understanding of what behaviors persist through the invasive process and how the invasive process acts as a selective agent to change behaviors that lead to successful invasions could help future efforts to identify invasive threats before those risks become realized.

Recently, it has been proposed that an ‘invasion syndrome’ may be a better indicator of potential invasive success than measures of singular behaviors in the case of unintentionally introduced species (Chapple et al 2012). Chapple et al. (2012) define an invasion syndrome as a specific behavioral syndrome, or group of correlated behaviors, that benefits populations over multiple stages of invasion (transportation, introduction, establishment, and spread), separated by barriers that need to be overcome to progress to the next stage (see Figure 2.1, see also Blackburn et al. 2011). Additionally, the ‘invasion syndrome’ theory predicts that the invasive stages and barriers between them will act as a filter for specific behavioral phenotypes, thereby decreasing overall phenotypic variation within invasive populations. High levels of directional selection for these specific traits may lead to a genetic erosion, resulting in a lack of phenotypic variation and the appearance of a specific, favored behavioral phenotype (Chapple and Wong 2016). However, this leads to a conundrum. By definition, a behavioral syndrome involves the maintenance of individual variation in behavioral types (Sih et al. 2004). If an invasive syndrome is to exist at all, the invasive population in question must exhibit some level of behavioral variation. Additionally, some models have shown that likelihood of invasive success could be improved when the population in question starts with a wider array of behavioral phenotypes (Fogarty et al. 2011). Part of this increased success may be due to the behavioral variation acting as a buffer against the strong directional selection for specific traits, slowing the decrease of phenotypic variation within the population.

Stages of Invasion

Studied Populations

Transport + Introduction

Barriers to pass this stage: fish must survive captivity and cultivation (in this case, artificial selection for specific colors).

Breeding Pool

Hybrids

Fish are moved from native habitats to breeding pools, hybridized.

Escape Pools

Hybrids

Fish accidentally escape to exotic location, potential introduction event.

Pet Trade

Hybrids

Fish are cultivated/bred and moved for sale to pet trade. Moved from breeding pools to pet trade for sale. From the pet trade, fish are released accidentally and intentionally.

Establishment

Barriers to pass this stage: fish must survive and reproduce after introduction to exotic environment.

Invasive Hawaiian Location

Hybrids

Fish have survived in location for generations. Population supplemented by continual release. Population experiences predation pressures.

Spread

Barriers to pass this stage: fish must survive and disperse to new

Upstream Populations

Hybrids

Fish move on from intial sources of intentional introduction further upstream. Processes of establishment and spread begin again.

Figure 2.1. A flowchart illustrating how the populations studied (dark grey) and barriers they faced in this system, adapted from Deacon and Magurran (2016) to include pet trade and intentional introductions (black); populations not studied are in light gray

Some recent studies have shown that individual variation of specific behaviors (such as aggression, boldness, exploration, or sociability) can play an important role in the overall invasive success of a species. For example, individual variability can mediate personality-dependent dispersal. Variation of individual personality composition within populations of the invasive mosquitofish, *Gambusia affins*, influenced how far the fish would disperse (Cote et al. 2010). Cote et al. (2010) suggest a mix of behavioral types falling within the population's overall behavioral syndrome may allow differential responses from ecological factors; variation in response to the environment could contribute to the overall impact and success of an invasive species. In another example, the benefits of some behaviors, like aggression, depended on the stage of invasion. In the invasive western bluebird (*Sialia mexicana*) aggression levels peaked during the initial invasion and spread into Montana, but later decreased once the invasive population was successfully established within the study site (Duckworth and Badyaev 2007). Duckworth and Badyaev (2007) suggest aggressive males make good dispersers and poor parents, whilst less aggressive males provide more parental care, but are not as likely to be successfully invasive individuals. Without the variation of aggression at a population level within this species, the western bluebird would have faltered at either the establishment or the spread stage. These studies exemplify why behavioral syndromes and individual variability can both play key roles in invasive success, despite the fact that it is not often quantified.

I have taken steps within this study to address how the invasion process influences individual behaviors, behavioral variation, and behavioral syndromes in an intentionally introduced invasive species. I chose the hybrid swordtail, *Xiphophorus helleri-maculatus,* as a model system. *X. helleri* and *X. maculatus* are live-bearing fishes with a native range spanning from southern Mexico to northern Honduras. Scientists hybridized *X. helleri* with *X. maculatus* in the early 1920s for the study of melanomas (Walter and Kazianis 2001). As a side effect, the hybrids produced color variations not found in the either parent species and were thusly introduced to the pet trade. Once there, the *X. helleri-maculatus* hybrids were introduced to 31 countries worldwide (CABI 2018), starting with an intentional introduction to the Hawaiian Islands in 1922 for pest control (Brock 1960). Intentional introductions have continued in Hawaiian waters to this day (Englund 1999, DMD pers. obs.). These fish are considered a global pest species responsible for the decline of native flora and fauna in many countries (CABI 2018, Maddern et al. 2011, Englund 1999, Milton and Arthington 1984). While previous behavioral studies have considered unintentional introductions, *X. helleri-maculatus* hybrids provide the opportunity to investigate a system of intentional introductions while testing for the effects of the invasion process on the formation of an invasive syndrome across populations at different stages of the invasive process. Additionally, previous studies that were interested in invasive syndromes made comparisons at the same stage of the invasive process (i.e. across closely related species, Monceau et al. 2015, Pintor et al. 2008; different invasive species, Bertelsmeier et al. 2015; across different invasive populations at the same stage within a single species, Brodin and Drotz 2014, Cote et al. 2010; or has not empirically examined the sequential filtering of several stages of the invasion process, Chapple and Wong 2016). In contrast, I sampled: the initial stages of artificial selection (*X. helleri-maculatus* hybrids from breeding pools), fish that survived the breeding pools and transport to be placed in the pet trade (*X. helleri-maculatus* hybrids from pet store), and fish from an invasive population that have gone through the stages of being introduced and becoming established (*X. helleri-maculatus* hybrids from Kauai, Hawaii), see Figure 2.1. With these three populations, I examine how stages of the invasive process (Chapple and Wong 2016, Chapple et al. 2012) and/or hybridization may have influenced behavioral changes.

In this study I ask the following questions: (1) Does the invasive process act as a filter to change any one of the different individual behaviors (boldness, aggression, exploration) that may increase invasive success? I would expect to see changes in levels of boldness, aggression, and exploration due to the potential selective filters fish must go through during the stages of invasion and the pet trade (2) Does the invasive process change the level of variation in individual behaviors across stages of invasion? I expect to see a decrease in overall variation within individual behaviors in populations that have gone through more stages of the invasive process as hypothesizes by Chapple and Wong (2016). (3) Does the invasive process act as a filter to *change* the relationship between the different individual behaviors as hypothesized by Chapple et al. (2012) to produce an invasive syndrome? The behavioral syndrome I expect to find in the successfully established and invasive Hawaiian population would be between three behaviors (boldness, aggression, and exploration) previously identified in literature and models as “a potentially beneficial behavioral package for invasive species” (Chapple et al. 2012, Fogarty et al. 2011). If a syndrome was found in the Hawaiian invasive hybrids, but *not* detected in either the breeding pool hybrids or pet trade hybrids, I would categorize this as an ‘invasion syndrome’, indicating that one of the later stages of invasion (such as establishment) might have selected for the syndrome (see Figure 2.2).

	Breeding Pool Hybrids	**Pet Trade Hybrids**	**Hawaiian Hybrids**	**Potential Factors Producing Behavioral Syndrome (BS)**
A	No BS	No BS	BS	INVASIVE SYNDROME Introduction/ Establishment Stage
B	No BS	BS	BS	PET TRADE SYNDROME Cultivation Practices/Transportation Stage
C	BS	BS	BS	HYBRIDIZATION SYNDROME Cultivation Practices/Hybridization

Figure 2.2 Comparison of three hybrid populations, and situations that would allow or not allow classification of a ‘invasive syndrome’ in the *X. helleri-maculatus* hybrids. BS = ‘behavioral syndrome’, No BS = ‘no behavioral syndrome’

Material and Methods

Xiphophorus helleri-maculatus hybrids were sampled from breeding pools at Imperial Tropics in Lakeland, Florida, USA (N = 22, 32% males), from the pet trade, purchased from Fish N’ Stuff in Athens, Ohio, USA (N = 36, 17% males) and sampled from a wild, invasive population in Kauai, Hawaii, USA (N = 16, 63% males). Fish were housed individually in 18.9 liter ‘home’ tanks with refugia, and isolated for at least one month before testing began. Fish were tested for exploration of a novel environment, aggression towards a conspecific, and boldness under risk in that order to reduce the possibility of carryover effects (see Bell 2013). During the exploration test, focal fish was placed in a novel 75.7 liter tank divided into ten areas (externally) and monitored continuously for 300 seconds (s). Latency to begin movement, total movements,

movements to different area, and total number of areas used were observed. Exploration methods were modeled after similar behavioral methods used to study sticklebacks by Bell and Stamps (2004). A mirror test was used to measure aggression towards conspecific. Fish were kept in their home tank, as it is their established territory, and were presented with a mirror. Latency until first approach, number of approaches, and number of bites were observed continuously for 300s. Methods have previously been used in another swordtail species (see D'Amore et al. 2015). During the boldness test, fish were allowed to fed undisturbed in their home tank for 30s before being 'surprised' with a net entering the tank. Fish were 'chased' for 15s before removal of net, and then latency to emerge from shelter and latency to resume feeding were measured for 300s post netting as the indicator of boldness. Fish were given at least two hours between tests, and all tests were conducted within 24 hours to ensure fish remained in the same state for all three tests. Tests were then repeated after 90-100 days to ensure behavioral repeatability. Short-term repeatability was found to be an indicator of long-term repeatability in personalities in another species of swordtail fish, *Xiphophorus birchmanni* (Boulton et al. 2014).

Data Analysis

Data were analyzed in program R v.3.1.2 (R Core Team 2016). Behavioral latencies were adjusted by subtracting individual's score from highest possible score +1 to prevent 0 counts, and so high numerical scores were indicative of high behavioral expression. Behavioral data was not normal; nonparametric methods were used to analyze the data. Intraclass coefficients (ICC) were used to calculate repeatability of behavioral measures (Hayes and Jenkins 1997). ICC scores were calculated with the ‘ICC’ function from the ‘psych’ package (Revelle 2018). Measures with highest repeatability scores were selected to represent associated behaviors. Differences between sexes were then examined using Mann Whitney U tests with the ‘stats’ package (R Core Team 2016). No significant differences between the sexes were detected for boldness (W = 947.5, P = 0.321), aggression (W = 858, P = 0.871), or exploration (W = 999, P = 0.143). Sexes were combined for following analyses.

Differences in behaviors across populations were compared via ordered expectations using a Jonckeere test in the package ‘clinfun’ (Seshan 2017). A Dunn’s post-hoc test (Dunn 1964) corrected for a false discovery rate (Benjamani and Hochberg 1995) was then used via the ‘dunn.test’ package (Dinno 2017) to see how individual populations differed from one stage of invasion to the next. Differences in behavioral trait variance between populations were analyzed with two sample asymptotic tests using the ‘asympTest’ package (Coeurjolly et al. 2009). Pair-wise relationships between most repeatable behaviors were examined within each population and calculated with Spearman’s rank correlation. Values for the strength of relationships between behaviors (using Spearman’s rho) were compared across populations using Fisher’s z-to-r transformation (Oldham et al. 2018, Zar 1999) for independent groups with the ‘cocor’ package (Diedenhofen and Musch 2015). Finally, to visualize the level of similarity in ‘behavioral space’ for our selected behaviors across the four populations we used non-metric multidimensional scaling (NMDS) (Holland 2008, McCune and Grace 2002, Legendre and Legendre 1998). The NMDS plot was created using Gower's distance measure in the 'vegan' package (Oksanen et al. 2017), with ellipses based on standard deviations of point scores. NMDS axes loadings were retrieved with the 'scores' function within the same package.

Ethical Statement

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. All appropriate animal ethics approval and licenses were obtained (Ohio University IACUC Approval: 12-L-042).

Results

Latency to emerge post-attack (boldness), latency to approach conspecific (aggression), and latency to explore novel tank (exploration) were the most repeatable measures per behavior (ICC scores > 0.3; Table 2.1) and were therefore used to represent the three behaviors in analyses thereafter.

Table 2.1

Output from intraclass correlation (ICC) analysis, showing repeatability of behavior in *X. helleri-maculatus* populations

Behavior	**ICC Score**
Latency to Emerge Post-Attack (Boldness)	**0.340***
Latency to Feed Post-Attack (Boldness)	0.320*
Latency to Approach (Aggression)	**0.560***
Number of Approaches (Aggression)	0.290*
Number of Bites (Aggression)	0.011
Latency to Start Moving (Exploration)	**0.650***
Number of Movements (Exploration)	0.550*
Movements to Different Area (Exploration)	0.560*
Total Areas Used (Exploration)	0.550*

Bolded text indicates measures used in all other analyses. An asterisk indicates a P value < 0.05. Values on 0-1 scale, where 0 indicates no repeatability and 1 indicates perfect repeatability

I detected a significant increase in boldness (JT = 1363.5, P = 0.002), aggression (JT = 1364.5, P = 0.002), and exploration (JT = 1250.5, P = 0.029) scores in an ordered pairwaise comparison. However, upon examination with a post-hoc analysis, boldness scores were not significantly different between fish from the Breeding Pool population and the Pet Trade population. Fish from the Invasive population were significantly bolder than fish from the Pet Trade (Table 2.2A, Figure 2.3). Fish from the Breeding Pool population were significantly less aggressive than fish from the Pet Trade population, but fish from the Pet Trade and from the Invasive population did not differ significantly in aggression (Table 2.2B, Figure 2.3). Finally, exploration scores were not significantly different between fish from the Breeding Pool population and the Pet Trade population or between fish from the Pet Trade or the Invasive population (Table 2.2C, Figure 2.3).

Table 2.2

Dunn's post-hoc comparisons of populations representing different stages of the invasive process

A. Boldness		
	Z	P
Breeding Pool vs. Pet Trade	-0.995	0.16
Pet Trade vs. Invasive	-2.583	**0.007**
B. Aggression		
	Z	P
Breeding Pool vs. Pet Trade	-2.126	**0.025**
Pet Trade vs. Invasive	-1.442	0.075
C. Exploration		
	Z	P
Breeding Pool vs. Pet Trade	-2.138	0.049
Pet Trade vs. Invasive	-0.152	0.999

Z = Dunn's pairwise *z* test statistic. A significant P value = $\alpha/2$. $\alpha = 0.05$. Significant P values in bold.

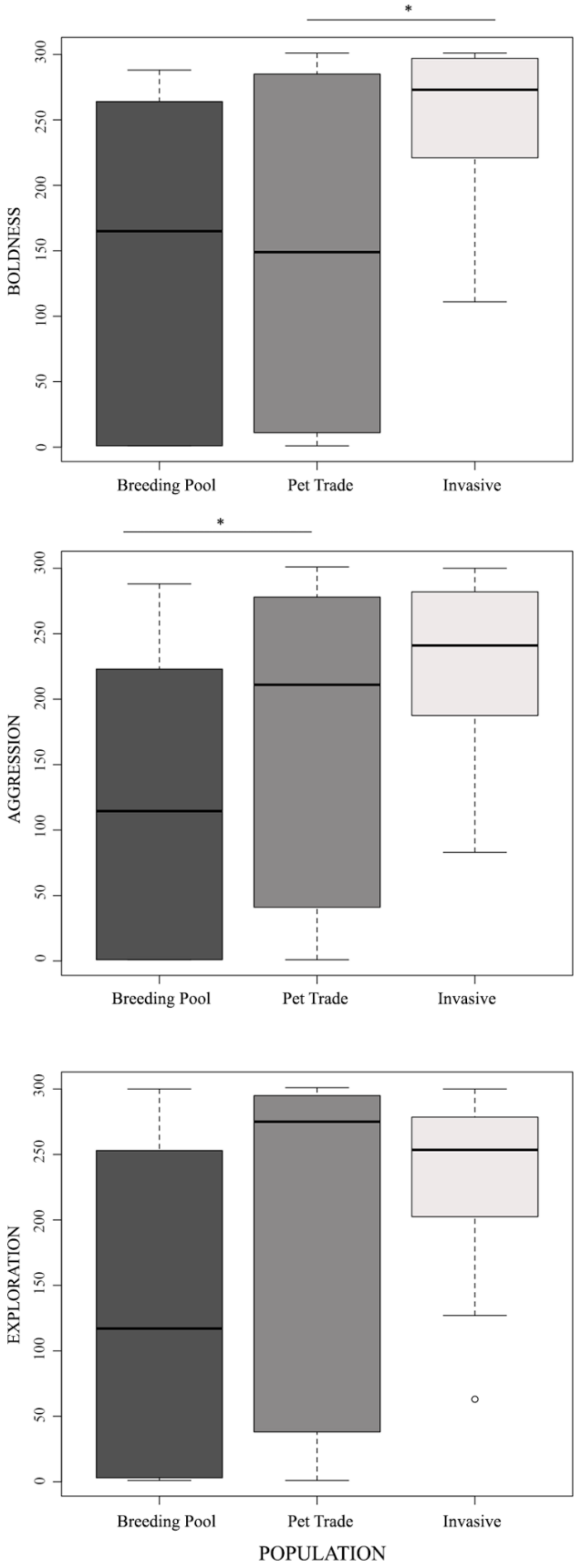

*
BOLDNESS
0
50
100
150
200
250
300
Breeding Pool
Pet Trade
Invasive
*
AGGRESSION
0
50
100
150
200
250
300
Breeding Pool
Pet Trade
Invasive
EXPLORATION
0
50
100
150
200
250
300
Breeding Pool
Pet Trade
Invasive
POPULATION

Figure 2.3 Variation within boldness, aggression, and exploration scores between hybrid populations: thick lines are median values, and boxes show the interquartile ranges. Significant differences indicated by bar and asterisk

I did not detect a decrease in variance between the Breeding Pool and Pet Trade populations in any of the three observed behaviors assessed. However, variance decreased in all three behaviors observed in the Invasive population as compared to the Pet Trade population (Table 2.3, Figure 2.3).

Table 2.3

Asymptotic test comparison of difference in variance across populations representing stages of invasion

A. Boldness		
	θ	P
Breeding Pool vs. Pet Trade	1.129	0.130
Pet Trade vs. Invasive	5.116	**<0.001**
B. Aggression		
	θ	P
Breeding Pool vs. Pet Trade	-1.520	0.936
Pet Trade vs. Invasive	4.259	**<0.001**
C. Exploration		
	θ	P
Breeding Pool vs. Pet Trade	-0.393	0.652
Pet Trade vs. Invasive	4.778	**<0.001**

θ = value of unified θ statistic. Significant P values in bold

While I detected significant pairwise relationships, the strength of these pairwise relationships varied between behaviors being analyzed (Table 2.4). While this variation also differed across populations, I did not detect a significant difference between these pairwise behavioral relationships when comparing these relationships across populations

(Table 2.5 see also Figure 2.4). Therefore, because the pairwise relationships do not appear to change across populations, the behavioral syndrome detected would not be classified as an invasive syndrome in the *X. helleri-maculatus* system.

Table 2.4

Pairwise analyses of behavioral correlations within populations using Spearman's rank correlation

		ρ	P
A. Exploration x Aggression			
	Breeding Pool	0.515	**0.014**
	Pet Trade	0.240	0.113
	Invasive	0.374	0.154
B. Exploration x Boldness			
	Breeding Pool	0.566	**0.006**
	Pet Trade	0.241	0.111
	Invasive	0.545	**0.029**
C. Boldness x Aggression			
	Breeding Pool	0.446	**0.037**
	Pet Trade	0.442	**0.004**
	Invasive	-0.052	0.850

ρ = Spearman's rho, significant P values in bold

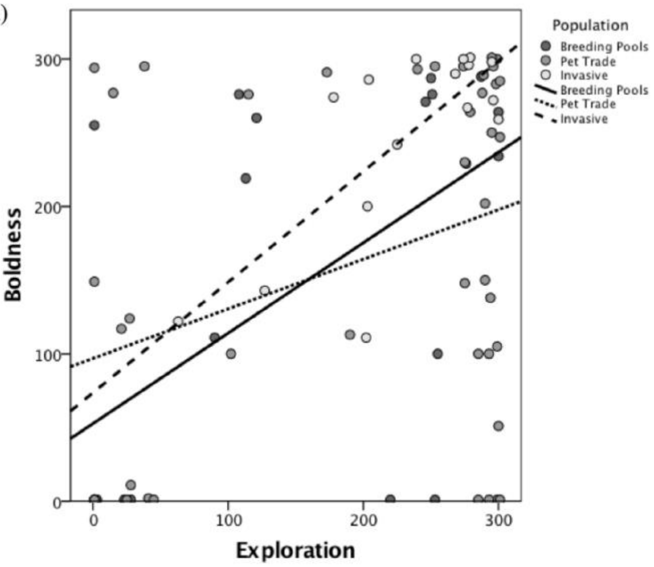
Table 2.5

Behavioral correlations do not change when compared across populations using Fisher's *r*-to-z transformation

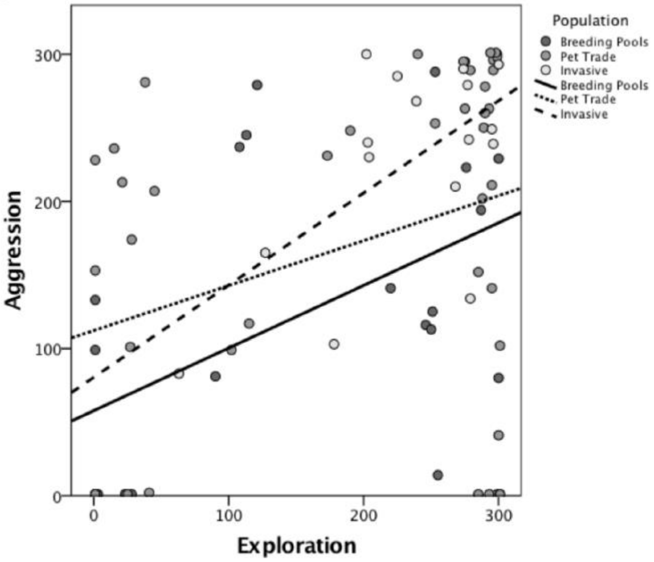
		Z	P
A. Exploration x Aggression			
	BP vs. PT	1.175	0.320
	PT vs. Inv	-0.467	0.954
B. Exploration x Boldness			
	BP vs. PT	1.432	0.250
	PT vs. Inv	-1.151	0.125
C. Boldness x Aggression			
	BP vs. PT	0.198	0.579
	PT vs. Inv	1.582	0.943

Z = Fisher's *z* score; BP = Breeding Pools; PT = Pet Trade, Inv = Invasive. 'BP vs. PT' indicates comparisons of correlations between these two populations, etc.

A)


Population
Breeding Pools
Pet Trade
Invasive
Breeding Pools
Pet Trade
Invasive
Boldness
0
100
200
300
Exploration
0
100
200
300

B)


Population
Breeding Pools
Pet Trade
Invasive
Breeding Pools
Pet Trade
Invasive
Aggression
0
100
200
300
Exploration
0
100
200
300

C)

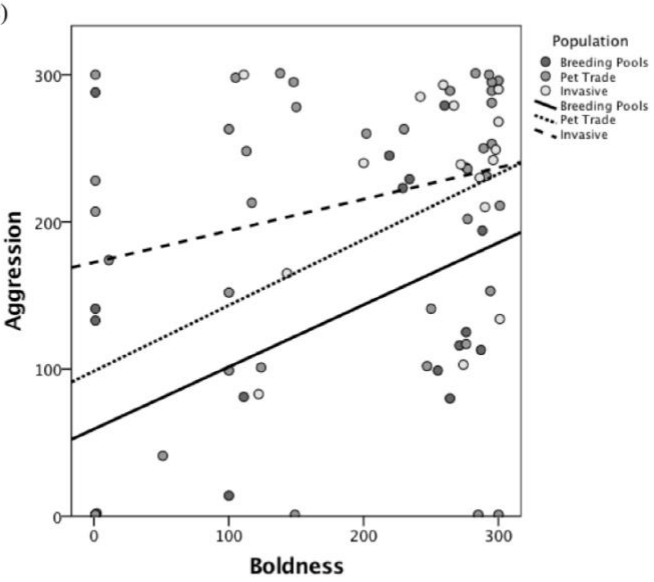

Population
Breeding Pools
Pet Trade
Invasive
Breeding Pools
Pet Trade
Invasive
Aggression
0
100
200
300
Boldness
0
100
200
300

Figure 2.4 Comparative plots of pairwise behavioral relationships between the three hybrid populations

All three populations were visualized in ‘behavioral space’ using an NMDS plot (Figure 2.5). Exploration loaded most highly on the X axis (NMDS 1) while aggression loaded most highly on the Y axis (NMDS 2); boldness did not appear to load on either of the first two axes (Table 2.6). Significant differences in behavioral variance between the pet trade and invasive hybrids (Table 2.3) can be observed in the different size and shapes of the ellipses. Populations within the pet trade and breeding pools have a much broader range across all behaviors, whereas the invasive population has a narrower range, nested inside these other two populations.

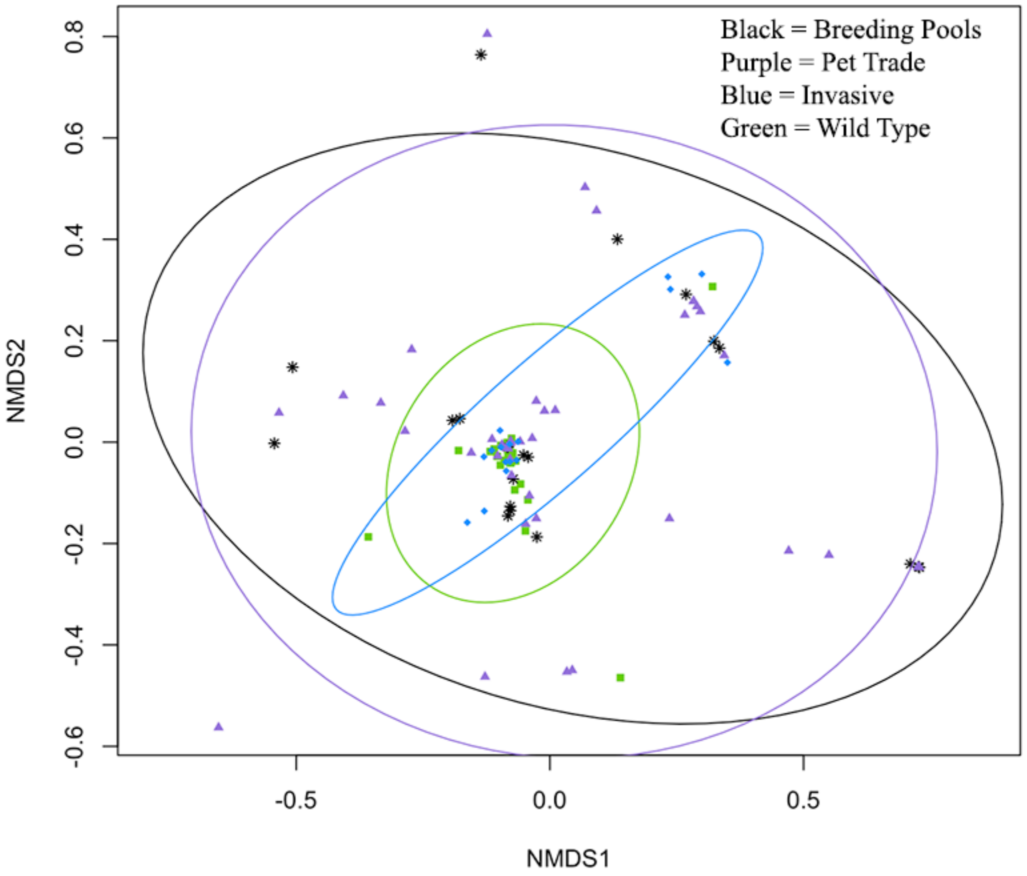


Figure 2.5. Non-metric multidimensional scaling (NMDS) used to represent hybrid swordtail populations in behavioral space

Table 2.6

Behavioral loadings on first two axes of non-metric multidimensional scaling (NMDS)

	NMDS 1	NMDS 2
Boldness	-0.3513	-0.2694
Aggression	-0.1130	0.3103
Exploration	0.3439	-0.0795

Discussion

Changes in individual behaviors and increases in behavioral variation, caused by either the invasive process or potentially hybridization, appear to play a role in the success of *X. helleri-maculatus* as an invasive species. While some of our results in this system of intentional introductions did not match the expectations for changes in behaviors for unintentional introduction systems, I did detect a reduction of behavioral variance in the Invasive population and increased boldness and aggression as predicted. First, moving through the invasive process seemed to increase boldness between the Pet Trade to the Invasive population, and aggression between the Breeding Pool to the Pet Trade population. Additionally, I detected a reduction in behavioral variance between the Pet Trade and Invasive populations, suggesting this reduction may have occurred during the later stages of invasion. While I detected these predicted changes in behaviors and behavioral variance between populations at different stages of the invasive process, I did not detect evidence for the formation of an invasive syndrome, as there was no significant difference in the pairwise behavioral relationships of the populations examined. I discuss possible reasons between the mismatch of our results for this intentionally introduced invasive species and predictions for unintentional invasion events, as well as examine potential reasons for the maintenance of the bold-aggressive-exploratory behavioral syndrome across the three observed populations. In addition, I will evaluate the implications of our results in relation to the success of *X. helleri-maculatus* hybrids as an invasive species below.

The comparisons of individual behaviors across populations at different stages of the invasive process do not meet our expectation of significant differences between. I only observe two significant differences: Invasive fish were bolder than the Pet Trade fish, and Pet Trade fish were more aggressive than the Breeding Pool fish. While I would expect boldness to increase through the stages of invasive, resulting in the Invasive population being the boldest, I would expect to observe the same pattern in aggression. Though the median score of aggression is higher in Invasive fish than Pet Trade fish (Figure 2.3), it is not significant (Table 2.3) – however, it is possible that a small Invasive population sample size is responsible for this negative result. Additionally, I did not detect a significant difference in variation in either pairwise population. It is possible that exploration is not as important for intentional introductions as it is for accidental introductions. Chapple et al. (2012) suggest that increased exploration may be important in the early stage of accidental introductions, in which animals must be 'taken up' before they can be transported. As this system represents one of intentional introductions, it is possible the earlier stages of invasion do not select for or against exploratory behaviors. Additionally, the invasive population sampled was at a site where fish were continually dumped (DMD pers. comm.); the more exploratory individuals may have already dispersed further upstream (which I was unable to sample), and thus left the 'less' exploratory individuals behind. These factors likely contribute to the lack of difference detected between fish from the breeding pool and Pet Trade populations, as well as the Pet Trade and Invasive populations. It is possible that the hybridization of *X. helleri* and *X. maculatus* produced this change in variation.

I expected to see a decrease in overall variation within individual behaviors in populations that had gone through more stages of the invasive process. Instead, I found no significant difference in variance in any behavior between the Breeding Pool and Pet Trade populations, but I did find a decrease in variance in all three behaviors when comparing the Pet Trade to the Invasive population. I suspect that original source of variance may be a result of the hybridization between *X. helleri* and *X. maculatus,* and that the lack of environmental selection in the fish experience within the breeding pools and pet trade allowed this variance to persist. Past studies have shown that hybrids between strains of Atlantic salmon (*Salmo salar*) showed intermediate expression of boldness and growth rate when compared with farmed and wild strains of Atlantic salmon (Einum and Felming 1997). The production of intermediate phenotypes and the lack of environmental pressures that would usually select against them (i.e., predation, limited resource availability), may account for some of the large changes in both the levels of individual behaviors and the variability within behaviors seen between the Breeding Pool, Pet Trade, and Invasive populations.

The overall decrease in variance in the invasive Hawaiian population can also be seen in the behavioral space; the invasive population has a much smaller ellipse than either of the other hybrid populations (Figure 2.5). This decrease in variance could be due to the later stages of the invasive process acting as a filter and selecting for specific, favorable phenotypes as predicted for unintentional invasive systems (Chapple and Wong 2016, Chapple et al. 2012), which Chapple and Wong (2016) also suggest may act on species in intentional invasive systems. It is also possible that being re-exposed to environmental pressures, such as predation, interspecific competition, and resource availability, limits variability of behavioral phenotypes by selecting against those intermediate behavioral phenotypes and drives the invasive hybrid population in a specific direction.

Finally, the comparisons across the three populations at different stages of the invasive process suggest that a behavioral syndrome represented by positive pairwise correlations between boldness, aggression and exploration was detected in the Breeding Pool population (Table 2.4). The Spearman's rho correlation values (ρ) were similar. Though the strength of ρ appears to differ between populations for any given pairwise comparison, I did not find any of the relationships to be significantly different when compared to one another with Fisher's r-to-z transformation (Table 2.5). Given that these correlations appear to have persisted through selection within the pet trade, transportation, multiple release events, and establishment of the hybrid population in Hawaii, I suggest that the behavioral syndrome in this system is not an "invasive syndrome", as it does not meet the criteria I discussed about (see Figure 2.2). I would like to note, however, that the Spearman's rho correlation does appear to be different (see Table 2.4, Figure 2.4) and even change direction in one case -- it is possible a small sample size of the invasive Hawaiian population may have led to this lack of detection of a significant difference. This should be readdressed in future studies to determine if sample size played a role, or if the syndrome is indeed stable across stages of the invasive process.

There are several potential reasons for the stability of the behavioral syndrome (bold-aggressive-explorative) I detected, as well as potential mechanisms that function to link at least two of the behaviors I examined (boldness and aggression). An adaptive hypothesis for the maintenance of the bold-aggression behavioral syndrome, is that the relationships between these behaviors is advantageous even in the face of selection to change the levels of the individual behaviors in the invasive environment. Additionally, these benefits may outweigh the costs to unlink the behaviors if produced by a proximate mechanism such as hormonal triggers (Bell and Stamps 2004), or if these behavioral traits are undergoing correlational selection. I previously detected a bold-aggressive behavioral syndrome in the related species *X. multilineatus*, and suggested that maternal investment was hormonally linking the two behaviors in the sons of mothers reared on high quality diets (D'Amore et al. 2015, see Chapter 1). The *X. helleri-maculatus* hybrids are likely being fed a high quality diet within the breeding pools and pet trade (reviewed by Tamaru et al. 2001), and these maternal effects may contributing to the positive relationship I detected between behaviors within the Pet Trade and Breeding Pool populations.

Behavioral syndromes may be lost through the reduction of behavioral variation due to strong selection (Chapple and Wong 2016). Strong selection for particular behavioral phenotypes during the invasive process has been detected in a variety of species (e.g. signal crayfish, *Pacifastacus leniusculus*, Pintor et al. 2008; cane toads, *Rhinella marina*, Lindstrom et al. 2013; house sparrows, *Passer domesticus*, Liebl and Martin 2014). Strong directional selection for specific behavioral phenotypes has even been indicated as a barrier to invasive range expansion in one case (e.g. invasive wasp, *Vespa velutina*, Monceau et al. 2014). Therefore, the maintenance of the behavioral syndrome in our system could indicate the lack of strong selection on the individual behaviors which would have led to reduced variation. I did not examine an invasive population post dispersal (spread), and future studies are needed to determine whether the relationship between behaviors, as well as the behavioral syndrome, change following the final stage of invasion. Finally, a hypothesis for the maintenance of the syndrome between the pet trade hybrids and the Hawaiian hybrids is that there has been a relatively constant input of new individuals (deliberate releases, DMD pers. obs.) into the Hawaiian population, located near the mouth of the river close to human populations. This influx of individuals could maintain the correlations between behaviors, as well as high intra-population variation in behaviors by introducing new individuals with the boldness-aggression-exploration syndrome. Additionally, this influx of individuals may influence more than one generation. The occurrence of a behavioral syndrome in *X. multilineatus* males was influenced by the mother their diet was reared on, not the resources she was using at the time of pregnancy or birth (D'Amore et al. 2015, see Chapter 1). The maternal investment of fish reared on high quality diets before release from the pet trade may continue to produce offspring with the positive correlations I observed, which would maintain the presence of the syndrome in the feral, invasive population for longer periods of time than if it were not being supplemented by new releases.

The *X. helleri-maculatus* system has provided some insights into how the interaction between hybridization and the invasive process may shape changes in behavior, and how this in turn may play a role in invasion success. To distinguish between the influence of hybridization and the invasion process itself as a selective filter on the changes in behavior and behavioral variation I detected, further testing of the hybrid fish in breeding pools prior to being artificially sorted, and of hybrid fish in the pet trade (post-sorting and distribution) are needed. Hybridization has been previously shown to change behaviors in fish. For example, juvenile rainbow (steelhead) trout (*Oncorhynchus mykiss*) from hybrid strains grew faster and were bolder than the non-hybridized strains (Johnsson and Abrahams 1991). Hybrid Atlantic salmon strains were intermediate between paternal strains in growth rate and boldness (Einum and Feling 1997), and hybridized cutthroat trout (*O. clarkii-mykiss* crosses) were intermediate in size and age at juvenile emigration (Kovach et al. 2014). In both of these cases, the overall variation in behavioral types was increased. Though there are many different factors that can explain the role of hybridization in the success of invasive fish species (replacement, competition, Huxel 1999; reproductive success, Rosenfield et al. 2004; hybrid vigor, Seiler and Keeley 2007), it is important to note that competitive superiority of hybrids is often due to behavioral differences (Oke et al. 2013). While these examples illustrate how important hybridization can be to understanding the success of invasive species, our results suggest changes in behavior or behavioral variation due to hybridization may be important to understand prior to the final stages of invasion and may exist well before species are introduced to exotic locations.

Lastly, it is important to consider how both early and later stages of invasion can change behaviors (as noted by Chapple et al. 2012), and whether the early stages of invasion are important in both deliberate and unintentional introductions. Given the need to understand the role of behaviors in the success of an invasive species to address a growing invasion problem, and to help direct intervention and prevention efforts, future studies of this system should focus on teasing apart hybridization from selection processes in the early stages of invasion, as well as focus on populations that have overcome the barriers of dispersal.

CHAPTER 3: MORE MELANIC INDIVIDUALS DO NOT MAKE ‘BETTER INVADERS’: MELANIN, INVASIVE BEHAVIORS, AND REPRODUCTIVE POTENTIAL ACROSS PET TRADE LINES OF *XIPHOPHORUS HELLERI-MACULATUS* HYBRIDS

Abstract

About one third of the world’s worst aquatic invaders had origins in the aquarium trade. While it is unlikely that all transport and release of aquarium species can be avoided, I investigated the hypothesis that certain morphological characteristics, such as color or body size, may indicate more invasive individuals or lines that could pose a higher risk of invasive potential. I analyzed three lines of the popular aquarium fish green swordtails (*Xiphophorus helleri-maculatus* hybrids), to determine if line, proportion of melanin or male size was correlated with traits known to increase invasive success -- the behavioral traits of aggression, boldness, exploration, and female reproductive potential. Given the genetically influenced size classes of males in swordtails, I also considered if male body size was correlated with these three behaviors. The results suggest that the most melanic line of fish was bolder and more explorative, and that proportion of melanin across lines was positively correlated with aggression, boldness, and exploration. Female reproductive potential was negatively correlated with proportion of melanin. Male size was correlated with exploration but not aggression or boldness. Our results suggest that while the most melanic fish exhibit more ‘invasive’ behaviors, the lowered fecundity likely prevents them from being more invasive than the other morphs investigated. In

addition, removing large males from the pet trade could potentially influence the extent to which populations spread from their initial sites of release.

Introduction

About a third of the world’s worst aquatic invasive species are popular aquarium or ornamental species, readily available to everyday consumers across the globe in commercial pet stores, local aquarium stores, and online retailers (Patoka et al. 2016, Padilla and Williams 2004). These invasive species can reach their final destination through a variety of means, from intentional stocking or purposeful release of household aquariums, to accidental escape from breeding pools, bait buckets, or garden ponds (Patoka et al. 2016, Tuckett et al. 2016, Strayer 2010, Gertzen et al. 2008). While aquaculture and aquarium release are both important pathways for the introduction of aquatic invasive species (Strayer 2010, Padilla and Williams 2004), most releases do not become successfully established populations, and fewer still become classified as ‘pest populations’ or ‘pest species’ (Gozlan 2008). The ‘tens rule’ suggests that only 1 of 10 introduced species will establish and only 1 of 10 of these will actually become invasive (Williamson 1996). Some of this variation in invasive success may be due to propagule pressure, which is the sum of all individuals released over introduction events of the introduced species (Blackburn et al. 2015, Simberloff 2009). However, variation in invasive success may also be influenced by specific behaviors that could be beneficial for invasive species, such as exploration, aggression, or boldness (Chapple et al. 2012). Behavior dictates how an animal will interact with both the biotic and abiotic factors of its new environment. Behavior can also define how an animal or population progresses through the various stages of the invasive process. For example, if individuals of a population have low propensity to disperse, the population likely will not succeed as an invasive species – even if it manages to become established, the population will never spread, thus failing to meet the criteria of an invasive species (see Chapple et al. 2012, see also Blackburn et al. 2011). Considering behavioral traits while examining invasive biology has led to better understanding, and in some cases, predictability, of invasive success (Chapple and Wong 2016, Blackburn et al. 2009).

Despite the increased understanding of the factors that lead to a successful invasive species, undesirable human behavior involving invasive species (i.e., the intentional release of an aquarium into a local waterway) continues to be difficult to predict or control (Gertzen et al. 2008, McNeely 2005) despite various attempts at shaping these particular actions through legislation and enforcement (Drake et al. 2014). While some researchers have suggested additional education, to be provided when a customer purchases a non-native species (Patoka et al. 2016, Lodge et al. 2006, Lodge et al. 2000), there is no universal agreement as to whether or not this is an effective method to combat the irresponsible behavior of aquarium owners. Instead, some researchers have suggested the need for stricter, more-inclusive legislation on the aquaculture industry or enforcement measures in combination with or instead of consumer education (Drake et al. 2015, Lodge et al. 2006).

While it is likely impossible to completely halt the accidental or intentional introduction of non-native species, and it is equally as unlikely to prevent the aquaculture industry from providing non-native species to the pet trade, perhaps it is possible to use easily identifiable morphological traits, such as color or size, to discourage the sale of particular individuals that may have higher ‘invasive potential’ than the average individual of a given species. Such a compromise would pose less of a threat to the livelihood of individuals within the aquaculture and aquarium industries, while at the same time decreasing the chances of a species’ invasive success. In addition to examining correlations between morphological traits and behaviors known to increase invasive success, it would be important to determine if the reproductive potential of some lines or morphologies are greater than others, as morphs with greater reproductive potential could pose more risk in the sense of propagule pressure. To assess whether such a compromise would be meaningful, I set out to determine if identifiable morphological traits – such as body color or size– were associated with any of the three behaviors most predictive of invasive success (aggression, boldness and exploration, see Chapple et al. 2012), or with reproductive potential in a popular aquarium fish, *Xiphophorus helleri-maculatus* hybrids.

Xiphophorus helleri-maculatus hybrids are a small, freshwater, live-bearing fish. The parent species, *X. helleri*, the green swordtail, and *X. maculatus*, the southern platyfish, have a native range spanning from southern Mexico to northern Honduras, but have not been documented to hybridize naturally (Rosenthal and García de León 2011, Clark et al. 1954). The *X. helleri-maculatus* hybrid was created in the early 1920s for the study of melanomas (Walter and Kazianis 2001). Backcrosses of these hybrids produced color variants not found in either parent species, while males still retained sword ornamentation (a sexually dimorphic trait found only in swordtail species) and were therefore popular with hobbyists when introduced to the pet trade. The first intentional introduction of 'green swordtails' (unknown if pure *X. helleri* or *X. helleri-maculatus* hybrids) occurred on the Hawaiian Islands in 1922 (Brock 1960). *X. helleri* and/or *X. helleri* hybrids are now found in 31 countries worldwide (CABI 2018) and are considered a global pest species responsible for the destruction of native and endemic flora and fauna species across many countries (CABI 2018, Maddern et al. 2011, Diggles et al. 2007, Morgan et al. 2004, Englund 1999, Courtenay et al. 1988, Milton and Arthington 1984).

Since introduction to the pet trade, several different 'lines' and color variants of swordtail hybrids are now commercially available. These variants are a result of two classes of color pigments commonly found in fish, carotenoids and melanin, as well as the structural colorations resulting in blue morphs. Melanin is responsible for brown, black and gray coloration and can be displayed in patches, spots, or bars (Price et al. 2008). Carotenoids are responsible for yellow, orange, and red pigments, and can be obtained from a fish's diet (Price et al. 2008). However, in the southern platyfish (*X. maculatus*), both carotenoids and pterins (a pigment less commonly found, but responsible for white, red, yellow, and orange pigmentation) are responsible for varying levels of red or orange pigmentation. (Basolo 2006). The mechanisms behind these coloration patterns in *X. maculatus* are not well understood, especially as some of these color patterns appear to be sex-linked, limited to specific populations, or the result of hybridization with other *Xiphophorus* species (Basolo 2006). Therefore, I did not compare lines with melanin expression to lines with red expression in this current study.

I chose to examine melanin coloration as an indicator of invasive success in the *X. helleri-maculatus* hybrids for several reasons. Proportion of melanic patches increase in *X. helleri-maculatus* hybrids as compared to the parental species (Walter and Kazianas 2001). Additionally, there is a positive relationship between the *Xiphophorus* melanoma receptor kinase (*Xmrk*) oncogene associated with melanin production in *Xiphophorus* fishes and increased male aggression in the Cortes swordtail, *X. cortezi* (Fernandez 2010). In another species of livebearer, the Eastern mosquitofish (*Gambusia holbrooki*), melanistic morphs also showed higher levels of aggression than silver morphs (Horth 2003). Aggression is often correlated with boldness and exploration, and aggression has been found to be positively correlated with boldness in the high-backed pygmy swordtail, *X. multilineatus* (D'Amore et al. 2015, see Chapter 1) as well as in *X. helleri-maculatus* hybrids (see Chapter 2). The melanocortin-4 receptor (*Mc4r*) gene is one of five melanocortin receptors responsible not only for the regulation of eumelanin and pheomelanin pigments, but also influences variation in both physiological and behavioral function within vertebrates, including the moderation of food intake and energy expenditure (Ducrest et al. 2008). Finally, there is the possibility that melanin is associated with reproductive potential, given that various hybridized strains of *X. helleri-maculatus* carry *Xmrk* and *Xmrk-2* (Schartl and Meierjohann 2011, Walter and Kazianis 2001), found to be associated with decreased viability and fecundity when it occurs naturally in *Xiphophorus cortezi* (Fernandez and Morris 2008).

Behavioral variation is likely a key component of invasive success, especially at the earlier stages of the invasion process (reviewed by Chapple and Wong 2016). Cote et al. (2010) found variation of behavioral types within populations of mosquitofish had an enhanced likelihood of invasive success as compared to populations composed of a singular behavioral type. Theoretical models (also using invasive mosquitofish) found populations with a mix of social and asocial individuals were more likely to spread than populations made up of primarily one type of individual (Fogarty et al. 2011). As higher levels of behavioral variance were detected in *X. helleri-maculatus* hybrids in the pet trade than hybrids from an establish invasive location (see Chapter 2), it would be important to know if one specific line of swordtail hybrid displays more behavioral variation than others.

Finally, I will examine the possibility that male size is indicative of invasive behaviors, such as aggression or exploration, which would suggest it would be worth considering a male fish's size when making decisions about which animals are 'safer' for the pet trade than others. The number of copies of the *Mc4r* gene on the Y chromosome regulates male size class within *Xiphophorus* fishes (Lampert et al. 2010). Overall body size is also important for female swordtail fish, as larger females have larger broods (Rios-Cardenas et al. 2013, Tamaru et al. 2001, Milton and Arthington 1983). However, females continue to grow after sexual maturity, and thus differences in the sizes of females across lines or morphologies may be less predictive of future reproductive output. Female platyfish (*X. maculatus*) do show significant variation in age at sexual maturity and overall egg availability once sexual maturity is reached (Kallman & Borkoski 1978), but to assess these differences, age at sexual maturity and age of the females would need to be considered. Therefore, I will determine if male body size plays a role in the invasive potential of a particular line or morphology, while controlling for female size when considering female reproductive potential.

I predict fish with a higher proportion of melanin will be more aggressive than fish with less melanin. Given that previous studies detected positive correlation between aggression-boldness in the swordtail species *X. multilineatus* (D'Amore et al. 2015, see Chapter 1) and *X. helleri-maculatus* hybrids found in both breeding pools and the pet trade (see Chapter 2), as well as between aggression-exploration and boldness-exploration in *X. helleri-maculatus* hybrids found within breeding pools (see Chapter 2), I expect more melanic fish to be more bold and exploratory in association with higher aggression. I expect to see lower behavioral variance in the more melanic Tuxedo line, as we expect to see selection for aggression, and therefore boldness and exploration. I expect smaller males to be more aggressive (as observed in *X. cortezi* males, Moretz 2003), and therefore bolder and more exploratory than larger males (via the positive correlations detected between behaviors in *X. helleri-maculatus* discussed above, see Chapter 2). Finally, while being more aggressive, bold, and/or exploratory could provide females with more resources, increasing reproductive potential, there is also the possibility that selection for increased melanin expression in females could lead to decreased viability and/or fecundity due to *Xmrk* (as observed in *X. cortezi*, Fernandez and Morris 2008). Therefore, I predict that female reproductive potential may not have a simple relationship with melanin coloration and could potentially depend on percentage of melanin.

Methods

Xiphophorus helleri-maculatus hybrids were obtained from a fish breeding operation that supplies pet stores (purchased from Imperial Tropics, Lakeland, FL, USA). Three lines were sampled from fish already sorted for sale to pet stores: Wild Type (N = 25), Spotted (N = 30), and Tuxedo (N = 30), see Figure 3.1. Fish were transported to a laboratory at Ohio University, and housed individually in 5-gallon ‘home’ tanks with refugia, and were isolated for at least two weeks before any testing began. Isolation allowed pregnant females to drop fry individually and allowed us to be certain of brood size, age, and mother identity. No female dropped more than one brood during the study period. Female behavior and morphology were measured at least one week after fry were dropped.

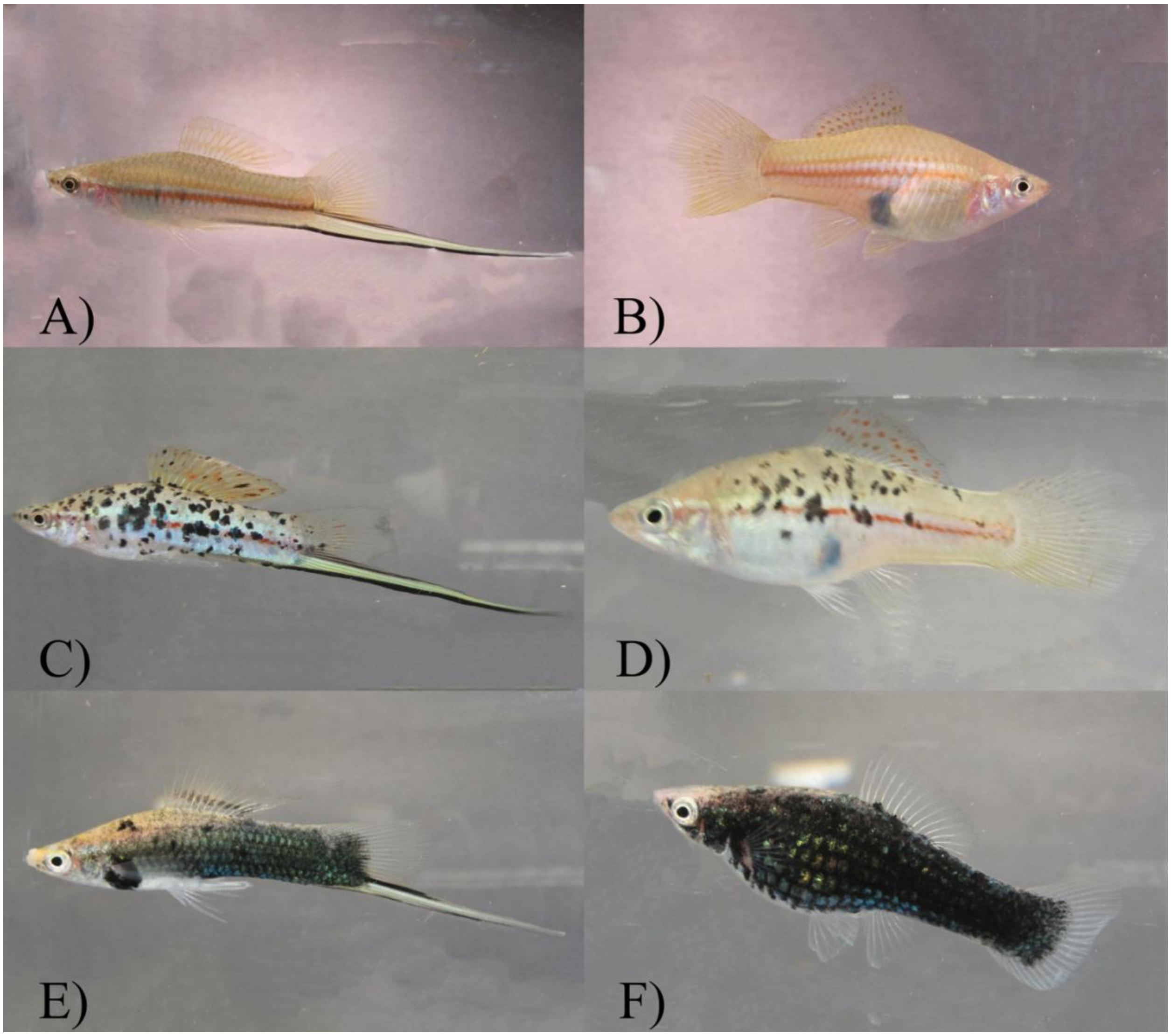


Figure 3.1 An example of Wild Type male (A) and female (B), a Spotted male (C) and female (D), and a Tuxedo male (E) and female (F). Melanic coverage varies even within lines, as illustrated with these samples.

Behavioral Tests

Fish were tested for exploration of a novel environment, aggression towards a conspecific, and boldness under risk in that order to reduce possible carryover effect (see Bell 2013). During the exploration test, the focal fish was placed in a novel 20-gallon tank externally divided into ten areas, and the individual was monitored continuously for

300 seconds (s). Latency to begin movement, total number of movements, movements to different area, and total number of areas used were recorded. Exploration testing was modeled after similar behavioral methods used to study three-spined sticklebacks (*Gasterosteus aculeatus*) by Bell and Stamps (2004), and has been used to study *X. helleri-maculatus* hybrids in a previous study (see Chapter 2). A mirror test was used to measure aggression towards a conspecific. Fish were kept in their home tank and were presented with a mirror. Latency until first approach and number of approaches were observed continuously for 300s. Methods have previously been used in another swordtail species, *X. multilineatus* (D'Amore et al. 2015, Chapter 1) and in *X. helleri-maculatus* hybrids (Chapter 2). During the boldness test, fish were allowed to feed undisturbed in their home tank for 30s before being 'surprised' with a net entering the tank. Fish were 'chased' for 15s before removal of the net. If fish took shelter during this time, the net 'hovered' near shelter for remaining time. Once the net was removed, the latency to emerge from shelter and latency to resume feeding were measured for 300s as the indicator of boldness. Boldness methods were designed for use with *X. helleri-maculatus* hybrids in a previous study (see Chapter 2). Fish were given at least two hours between all tests to reduce the potential of stress responses. All tests were conducted within 48 hours to ensure fish remained in the same 'state'. Repeatability was previously found within *X. helleri-maculatus* hybrids (Chapter 2) and short-term repeatability was found to be an indicator of long-term repeatability in another swordtail species, *X. birchmanni* (Boulton et al. 2014).

Melanic Morphology

To measure size, photos were taken in a small, glass V with a Canon PowerShot SD120015. Photos were measured using ImageJ 1.x (Schneider et al. 2012). Standard length, depth, body area, and area of melanin covering body were measured for all fish on both sagittal planes, and then averaged for a fish's score. Only melanin on a fish's body was measured – melanin that occurred on any fins was not measured. Percentage of melanin was calculated as area of melanin divided by total body area, per side. Amount of melanic coverage varied even among lines (see Figure 3.1, especially C-F), and so a quantitative measure was required alongside line classification.

Data Analyses

Data were analyzed with program R v3.1.2 (R Development Core Team 2016). Behavioral measurements previously determined to be the most repeatable were selected to represent each of the three behaviors (Chapter 2). Behavioral latencies were adjusted by subtracting individual's score from highest possible score +1 to prevent 0 counts, and so high numerical scores were indicative of high behavioral expression. All data were not normally distributed and statistical analyses were chosen accordingly. Differences between sexes within behavior and melanic coverage were analyzed with Mann-Whitney U tests (Mann and Whitney 1947). I did not detect any significant difference in boldness ($W = 900$, $P = 0.484$), aggression ($W = 869$, $P = 0.685$), exploration ($W = 959$, $P = 0.227$), or percentage of body covered by melanin ($W = 968$, $P = 0.186$) between the sexes, so the sexes were combined for further analysis. Differences in behaviors across populations were compared via ordered expectations, as I had a priori expectations that the lines would behave differently based on melanic coverage. a Jonckeere test in the package ‘clinfun’ (Seshan 2017). A Dunn’s post-hoc test (Dunn 1964) corrected for a false discovery rate (Benjamani and Hochberg 1995) was then used via the ‘dunn.test’ package (Dinno 2017) to see how individual populations differed from one stage of invasion to the next. I used two-tailed Mann-Whitney U tests to assess behavioral differences between females who did and did not produce offspring, but as I did not have any prior expectations for this comparison. Differences in behavioral trait variance between populations were analyzed with two sample asymptotic tests using the ‘asympTest’ package (Coeurjolly et al. 2009), and also included a priori expectations based on differences in behavioral variance expected across the lines. Finally, differences in brood size while controlling for female size across lines was analyzed with Mann-Whitney U tests, using a one-sided design based on prior expectations.

Variation in whether a female had offspring and variation in number of offspring females produced were analyzed with generalized estimating equations (GEEs) using the ‘geepack’ package (Halekoh et al. 2006, Yan and Fine 2004, Yan 2002). Variation in whether a female had offspring was analyzed with binomial models, including line, female’s overall size, length of time spent in the lab, and percentage of melanin as covariates. Analyses started with a model that included all possible predictor variables and then found the minimal adequate model. The assessment was done longhand by removing predictor variables that did not significantly impact the model in a step-by-step process until I arrived at the minimum adequate model. I then preformed an ordinal logistic regression and used the package ‘ggplot2’ (Wickham 2016) to plot the results.

Finally, pair-wise relationships between male size and behavior, between proportion of melanin and behavior, between proportion of melanin and female reproductive success, and between behavior and female reproductive success were examined across populations and calculated with Spearman rank correlation coefficients. The strength of these correlations were compared across populations using Fisher’s z-to-r transformation (Oldham et al. 2018, Zar 1991) for independent groups using the ‘cocor’ package (Deidenhofen and Musch 2015).

Results

Using an analysis via ordered expectations, I detected a trend of increasing boldness (JT = 1472.5, P = 0.013) and exploration (JT = 1461, P = 0.020) scores, but did not detect the same trend in aggression scores (JT = 1370, P = 0.085). Upon examination with a post-hoc analysis, the Wild Type population was significantly less bold than the Tuxedo population but not the Spotted population. The Spotted population was not significantly less bold than the Tuxedo population (Figure 3.2, Table 3.1A). The Wild Type population was not significantly less aggressive than either the Spotted or the Tuxedo populations, nor was the Spotted population significantly less aggressive than the Tuxedo population (Figure 3.2, Table 3.1B). The Wild Type population was significantly less exploratory than both the Spotted and the Tuxedo population. The Spotted population was not significantly less exploratory than the Tuxedo population (Figure 3.2, Table 3.1C).

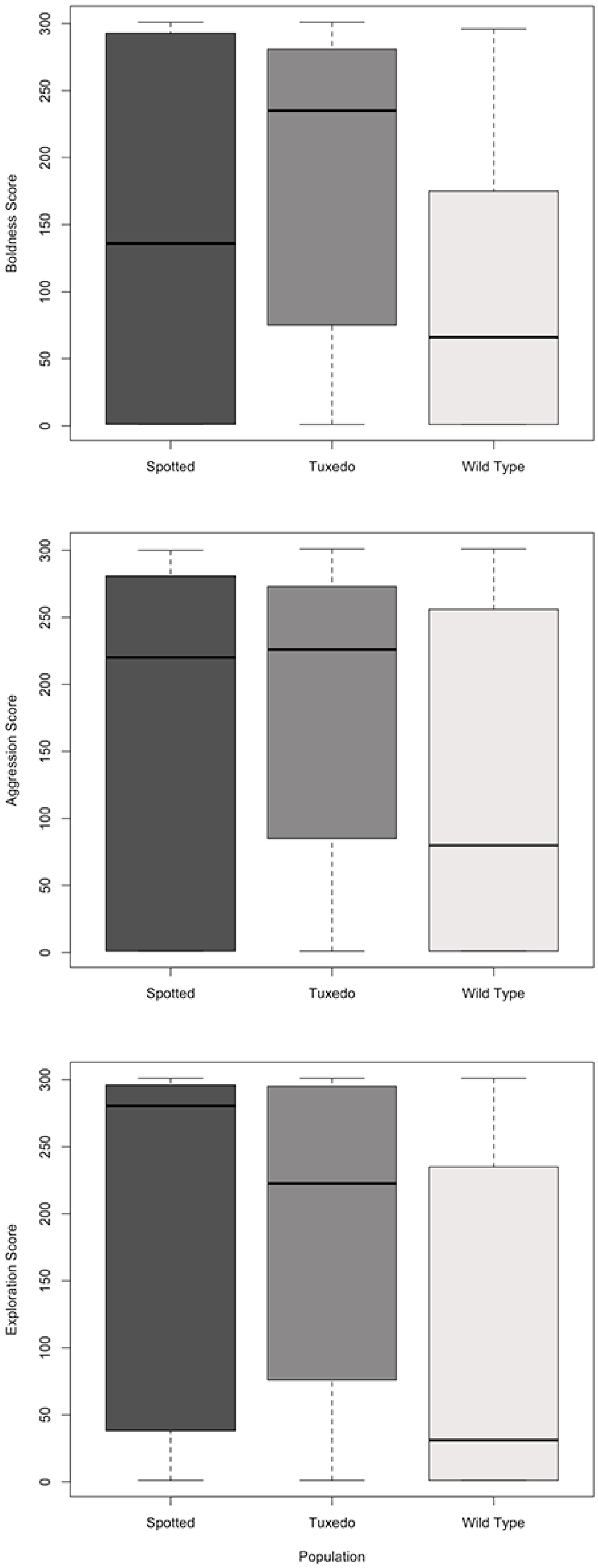

Boldness Score
0
50
100
150
200
250
300
Spotted
Tuxedo
Wild Type
Aggression Score
0
50
100
150
200
250
300
Spotted
Tuxedo
Wild Type
Exploration Score
0
50
100
150
200
250
300
Spotted
Tuxedo
Wild Type
Population

Figure 3.2 Differences in mean behavioral scores across populations. Higher adjusted scores represent a higher expression of the given behavior. Boxes: 25th and 75th percentiles; center line: median; 1.5 times the interquartile range of the data.

Table 3.1

Dunn's post-hoc comparisons across populations with varying amounts of melanin

A. Boldness	Z	P
Wild Type vs. Spotted	-1.453	0.110
Wild Type vs. Tuxedo	-2.441	**0.022**
Spotted vs. Tuxedo	-1.036	0.150

B. Aggression	Z	P
Wild Type vs. Spotted	-0.971	0.249
Wild Type vs. Tuxedo	-1.451	0.220
Spotted vs. Tuxedo	-0.503	0.307

C. Exploration	Z	P
Wild Type vs. Spotted	-2.89	**0.006**
Wild Type vs. Tuxedo	-2.318	**0.015**
Spotted vs. Tuxedo	0.600	0.274

Z = Dunn's pairwise *z* test statistic. A significant P value = $\alpha/2$. $\alpha = 0.05$. Significant P values in bold

I did detect a significant difference in the variance of boldness; fish from the Spotted population exhibited significantly higher variance than fish from the Tuxedo population (Table 3.2A). However, I did not detect any other significant differences in behavioral variance under the a priori expectations set (Figure 3.2, Table 3.2).

Table 3.2

Asymptotic test comparison of difference in variance across populations representing stages of invasion

A. Boldness	θ	P
Wild Type vs. Spotted	-2.965	0.999
Wild Type vs. Tuxedo	-0.639	0.739
Spotted vs. Tuxedo	2.102	**0.018**

B. Aggression	θ	P
Wild Type vs. Spotted	0.065	0.474
Wild Type vs. Tuxedo	1.536	0.062
Spotted vs. Tuxedo	1.446	0.074

C. Exploration	θ	P
Wild Type vs. Spotted	-0.193	0.577
Wild Type vs. Tuxedo	1.102	0.156
Spotted vs. Tuxedo	1.077	0.141

θ = value of unified θ statistic; significant P values in bold

When examining all individuals, percentage of melanin was positively correlated with boldness (Spearman rank correlation: $r_s = 0.273$, N = 85, P = 0.011), aggression (Spearman rank correlation: $r_s = 0.218$, N = 85, P = 0.045), and exploration (Spearman rank correlation; $r_s = 0.239$, N = 85, P = 0.028).

In pairwise comparisons between male body size and behaviors, body size was positively correlated with exploration (Spearman rank correlation: $r_s = 0.371$, N = 30, P = 0.044). However, male body size was not significantly correlated with aggression (Spearman rank correlation: $r_s = -0.141$, N = 30, P = 0.459) or exploration (Spearman rank correlation: $r_s = 0.189$, N = 30, P = 0.318).

Finally, reproductive potential (both number of females that dropped broods and brood sizes) was evaluated in relation to the three invasive behaviors, compared across lines, and considered in relation to percentage of melanin. Median boldness (W = 308, P = 0.369), aggression (W = 318, P = 0.470), and exploration (W = 329, P = 0.601) scores were not significantly different between females who reproduced (N = 20) and females who did not reproduce (N = 35). In addition, neither boldness (Spearman rank correlation: $r_s = -0.154$, N = 20, P = 0.517), aggression (Spearman rank correlation: $r_s = -0.118$, N = 20, P = 0.620), nor exploration (Spearman rank correlation: $r_s = -0.112$, N = 20, P = 0.639) scores were significantly correlated with number of offspring produced when controlling for female size. However, the Tuxedo line had significantly fewer offspring than the Spotted or Wild Type females when comparing all females and reproductive females only) when controlling for female size. Median brood size did not differ significantly between the Spotted and Wild Type females in either comparison (see Table 3.3, Figure 3.3).

Table 3.3

Pairwise comparisons of brood sizes across different lines of *X. helleri-maculatus* females

A. All Females	W	P
Wild Type vs. Spotted	160	0.365
Wild Type vs. Tuxedo	215	**0.012**
Spotted vs. Tuxedo	285.5	**0.011**

B. Reproductive Only	W	P
Wild Type vs. Spotted	29	0.235
Wild Type vs. Tuxedo	28	**0.003**
Spotted vs. Tuxedo	36	**0.001**

W = Wald test statistic; significant P values in bold

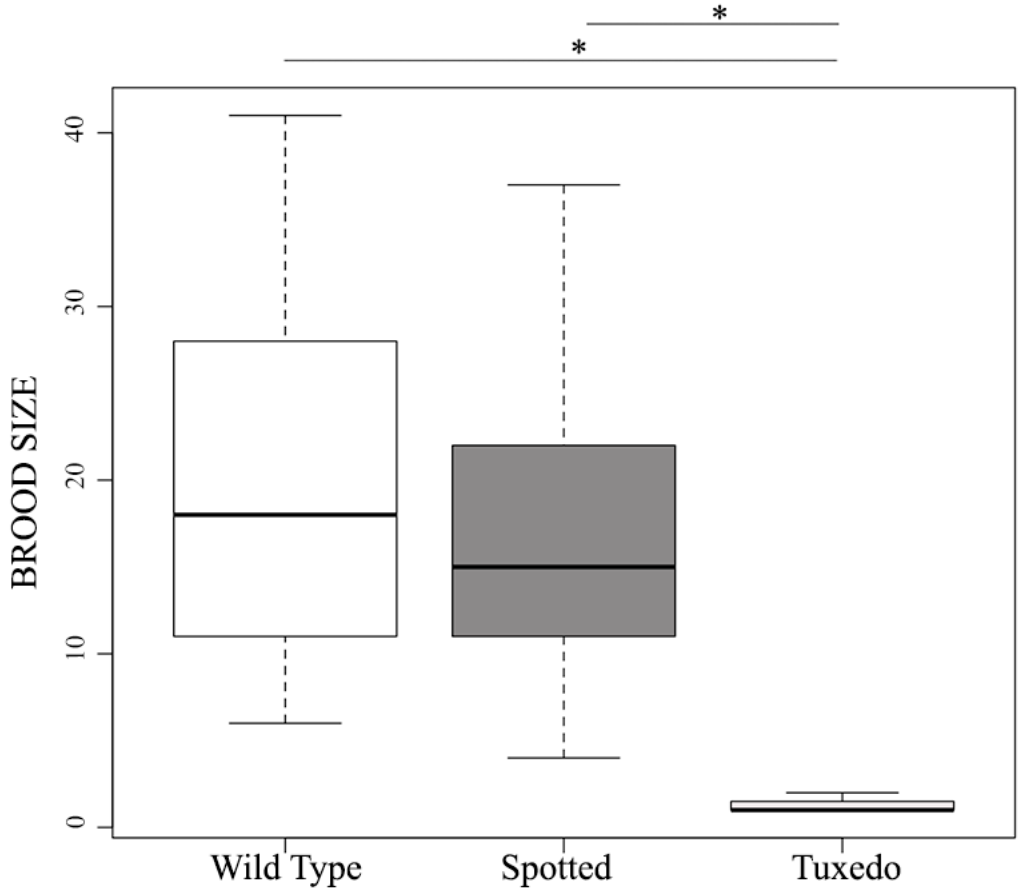


Figure 3.3 Differences in mean brood sizes of reproductive females across populations. Boxes: 25th and 75th percentiles; center line: median; 1.5 times the interquartile range of the data

Variation in a female's reproductive success was best explained with a model including only the percentage of melanin on her body (Table 3.4). As the amount of melanin increases on a female's body, the likelihood of reproductive success decreases (Figure 3.4). Additionally, while controlling for female size, percentage of melanin and number of offspring were negatively correlated (Spearman rank correlation $r_s = -0.672$, N = 20, P = 0.001).

Table 3.4

Total percentage of melanin indicative of reproductive success in all females in best fit generalized estimating equation

	Estimate	Std. Error	Wald	P value
Intercept	-0.048	0.368	0.020	0.897
% Melanin	-0.020	0.010	4.060	**0.044**

Wald = Wald test statistic; significant P values in bold

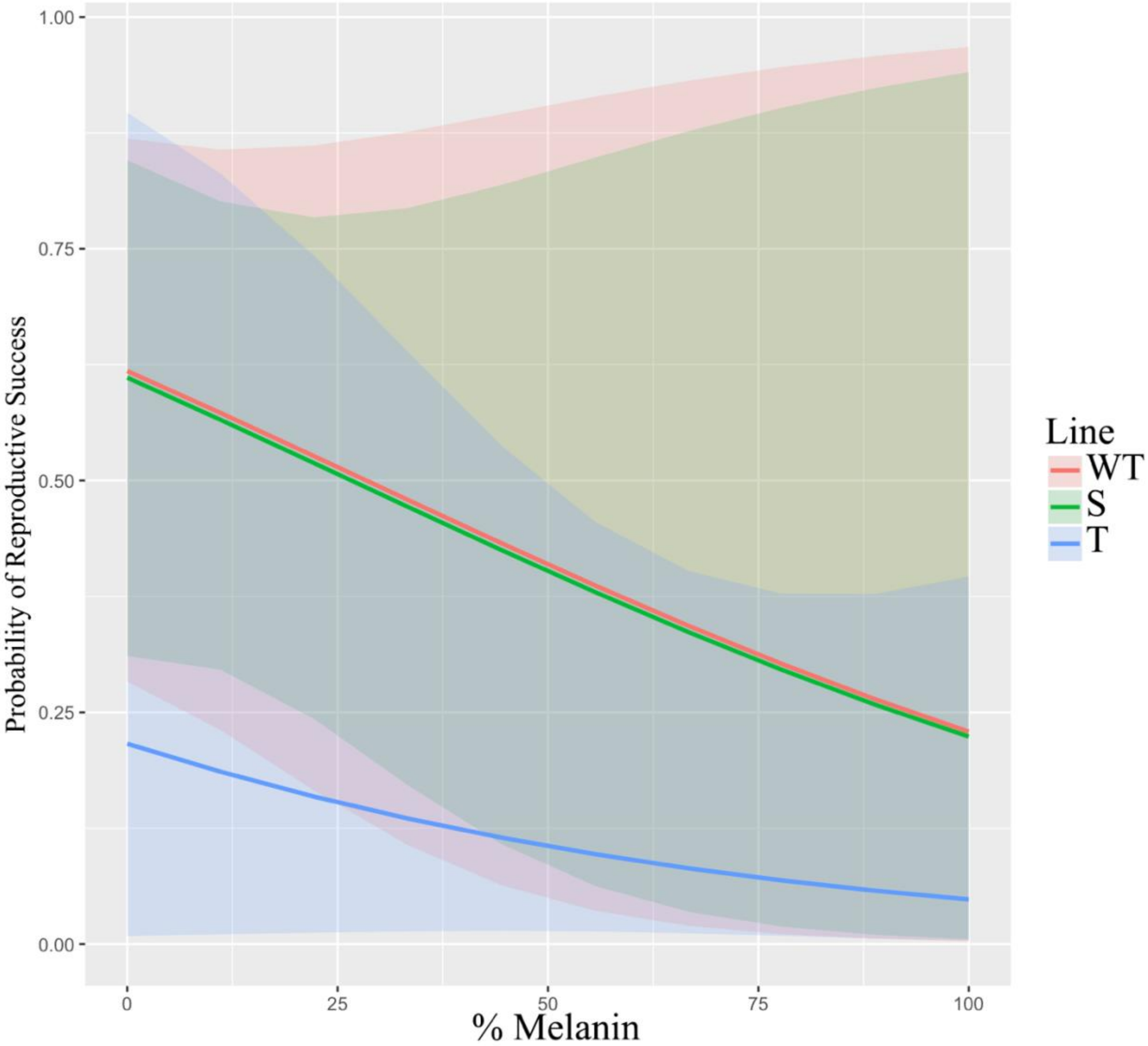


Figure 3.4 Predicted probability of successfully dropping a brood decreases as percentage of melanin increases, regardless of a female's line. Line: WT = Wild Type, S = Spotted, T = Tuxedo

Discussion

Melanin appears to play a role in both individual behavior and female fecundity in the green swordtail hybrid, *X. helleri-maculatus*. Coinciding with my predictions, I saw that increased melanin was positively correlated with higher aggression scores, as well as higher boldness and exploration scores across all individuals tested. However, there were no significant differences in aggression when comparing populations, and no significant behavioral differences between the Spotted and Tuxedo populations in exploration or boldness, which may indicate that an individual's proportion of melanin is more predictive of their behavior than the line they came from. With one exception in boldness, I did not see a decrease in behavioral variance as expected in the more melanic lines. I did detect a positive correlation between male size and exploration, but no significant correlations between male size and the other behaviors. Finally, I found that the number of offspring a female produced was not correlated with any specific behavior, but was correlated with her overall percentage of melanin. In addition, there was a significant difference in brood size across the three lines, with Tuxedo females having significantly smaller broods than the other two lines. Therefore, while a higher proportion of melanism seems to be indicative of more 'invasive' behaviors in *X. helleri-maculatus* hybrids, this advantage may be cancelled out by the significantly lower reproductive output observed in the most melanic females. Below, I discuss how lower fecundity may cancel out any gains afforded to the most melanic individuals with higher aggression, boldness, and exploration, as well as potential mechanisms for linking melanin to behaviors and lower fecundity. In addition, we will discuss the application of our results for informing consumers about the risks of releasing fish from the pet trade, with a focus on how populations of mixed morphs may have the most invasive potential.

The most melanic individuals also had the highest aggression, boldness, and exploration scores. While it is likely that the same mechanism that produces increased melanism is also increasing aggression in these swordtail hybrids (*Xmrk-2* gene, Fernandez 2010, Walter and Kazianis 2001), the associated increase in boldness and exploration is likely due to a behavioral syndrome or coping style, and may not be directly linked to the increased production of melanin pigmentation; a bold-aggressive syndrome has previously been detected in *X. multilineatus* (D'Amore et al. 2015, see Chapter 1), and a bold-aggressive-exploratory syndrome has previously been detected in both *X. helleri* and *X. helleri-maculatus* hybrids (see Chapter 2). Similar results have been observed in other vertebrate species. Darker male eastern Hermann's tortoises (*Eurotestudo oettgeri*) are both bolder and more aggressive than their lighter counterparts, but not more exploratory, leading authors to suggest that the increase in boldness may be an indirect consequence of the linkage between coloration and aggression in this species (Mafli et al. 2011). A similar pattern was also found in male siskins (*Carduelis spinus*), a highly social bird species, in which males with larger black bibs were more aggressive, but also displayed more traits associated with boldness, which the authors describe as a proactive 'coping style' in a species in which males are constantly challenging one another within social hierarchies (Mateos-Gonzalez and Senar 2012). Finally, darker male tree lizards (*Urosaurus ornatus*) were more territorial and dominant than their lighter counterparts (Zucker 1989). While Zucker (1989) did not classify these behaviors as bold or aggressive, it seems logical to associate higher levels of aggression and boldness with increased territoriality and social dominance. Therefore, it is possible that melanin influences behaviors associated with boldness in a similar manner that is associated with aggressive behaviors, as suggested by these results and those observed in melanic male eastern Hermann's tortoises (Mafli et al. 2011), more melanic male siskins (Mateos-Gonzalez and Senar 2012), and darker male tree lizards (Zucker 1989).

Overall male body size was not significantly correlated with either aggression or boldness, but was positively correlated with exploration, indicating that larger males were more exploratory. I had predicted that smaller males would be more aggressive based on results found in a study on another swordtail species, *X. cortezi*, (Moretz 2003), but did not find this to be the case in the current study. As all the fish I examined were collected from a breeding facility and had lived in social groups prior to isolation and testing, the lack of a relationship between size and aggression cannot be attributed to a lack of previous experience with aggression and dominance. Previous studies have shown that adult male swordtails (*X. helleri*) use not only the body size of their opponent, but also previous experience to gauge aggression; they also moderate contest initiation and escalation based on hierarchy and 'fighting ability' information accessed from eavesdropping on contests between other males (Earley et al. 2003, Earley and Dugatkin 2002, Beaugrand et al. 1996, Franck and Ribowski 1989, Franck and Ribowski 1987). Therefore, given that adult male size in swordtails has a strong genetic influence (Kallman 1989), removing males from the pet trade based on size may not reduce aggression or boldness, but removing larger males could reduce the probability that introduced fish would spread to additional areas (exploratory behavior) given the positive correlation I detected between male size and exploration.

Median behavioral scores did not differ significantly between females who did and did not drop a brood, nor were they significantly correlated with the number of fry produced. Instead, variation in whether or not a female reproduced was best explained by her overall percentage of melanin (Table 3.4, Figure 3.4). I also detected a significant difference in brood size while controlling for female size (Table 3.3, Figure 3.3). Females from the most melanistic Tuxedo population had significantly less offspring than either of the other two populations, which were not significantly different from each other. This is particularly interesting because melanic male morphs in other live bearing species of fish (Horth 2003) and across other vertebrate taxa (Ducrest at al. 2008) have shown an increased frequency in the use of mating behaviors, suggesting that the decrease in female fecundity of melanistic lines is not due to a lack of attempted copulations. A similar pattern has been detected in Carduline finches, where more melanic males sired fewer broods, and the clutches they did sire were smaller than those of less melanic males (Bókony and Liker 2005). In the hybrid *X. helleri-maculatus*, the mechanism for the relationship between melanin and lower female fecundity may involve the *Xmrk* gene (Weis and Schartl 1998). Pure *X. helleri* do not carry the *Xmrk* gene, but various hybridized strains of *X. helleri-maculatus* carry *Xmrk* and *Xmrk-2* (Schartl and Meierjohann 2011, Walter and Kazianis 2001). *Xmrk* has been found to be associated with decreased viability and fecundity when it occurs naturally in *Xiphophorus* fishes

(Fernandez and Morris 2008). *X. cortezi* females have a frequency-dependent preference for males displaying melanic patterning associated with *Xmrk* (Fernandez and Morris 2008), while *X. helleri* females prefer spotted males in turbulent streams, but not clear ones (Franck et al. 2001). I suggest that through the process of rigorous line-breeding used to maintain specific lines of fish, fish farmers may have circumvented female mate preference for (or against) more melanic males, resulting in lower fecundity across several generations of selective breeding. This specific form of breeding was used in the Tuxedo line, but was not required to maintain either the Spotted or Wild Type line (DMD pers. comm.)

The extent to which lower fecundity may cancel out any gains afforded to the most melanic individuals with higher aggression, boldness, and exploration needs further evaluation. Invasive populations with faster population growth rates (larger broods, more broods per productive season) and populations that produce more offspring in a new environment are more likely to spread more expansively in a new environment (Caswell et al. 2003). Increased reproductive output can be a factor that simulates increased propagule pressure – the more individuals that are introduced to an exotic location (be it through release or reproduction), the more likely that population is to spread (Blackburn et al. 2015). Therefore, it is interesting to note that no melanic morphs were observed or captured in invasive populations sampled in Kauai, Hawaii (pers. obs.) or in Western Australia (Maddern et al. 2011). In fact, Maddern et al. (2011) note that about 5% of fish sampled exhibited a melanic pigmentation on their caudal fins, which they consider an indication of hybridization. Total melanic pigmentation does not persist from one generation to the next in all offspring in the Tuxedo line within lab conditions (pers. obs.), and the classic backcrosses for *X. helleri-maculatus* hybrids for melanomas and macromelanophores produce a mix of offspring with and without melanin (Schartl and Meierjohann 2011, Walter and Kazianis 2001). Therefore, scarcity of melanistic individuals in exotic locations, even though they are prevalent in the pet trade, may indicate they are being selected against once released, either through female mate choice, lowered fecundity, or predation.

In conclusion, I did not find a specific morph or size that was 'more invasive' than the others in relation to both behaviors and fecundity. While the most melanic fish (from the 'Tuxedo' line) were more bold and exploratory, they also had the lowest reproductive output. Larger size classes of males may pose more problems in relation to the spreading from the site of introduction, but they were not more aggressive or bold. The release of multiple lines of swordtail hybrids may be the most problematic by virtue of mixed behavioral types providing the variation necessary to select for the most invasive individuals in the new location, a phenomenon that has been observed in the invasive Western mosquitofish, *G. affins*, and needs further exploration in the swordtail system. (Cote et al. 2010, Fogarty et al. 2011). More research is needed to understand the influence of linebreeding for morphological traits on behavior, specifically in animals found in the pet or aquarium trades, and how this may in turn affect invasive success if these animals are released. Specifically, further investigation is required to understand if linebreeding for other traits (such as carotenoid-based coloration) in *X. helleri-maculatus* has similar effects on behavior or female fecundity, or if the results observed above are related only to selection for melanin and associated traits.

CHAPTER 4: EVALUATING DIFFERENT LINES AND THE INFLUENCE OF LINE-BREEDING METHODS ON TRAITS ASSOCIATED WITH INVASIVE SUCCESS IN THE GREEN SWORDTAIL HYBRID, *XIPHOPBORUS HELLERI-MACULATUS*

Abstract

About one third of the world's worst aquatic invaders originate in the aquarium hobby. Livebearers represent a disproportionate amount of invasive freshwater fish species, likely due to their popularity as pets. I investigated whether differences in the line-breeding process used to sustain particular lines of *Xiphophorus helleri-maculatus* hybrids within the pet trade influenced traits that associated with invasive success. I compared lines that undergo different levels of selection, as well as populations that had experienced rigorous selection to populations that had yet to be processed through more rigorous selection procedure within lines. I did not find any significant behavioral or reproductive differences between lines that experience different levels of artificial selection, although one specific line (Sky Blue) did have higher reproductive potential. In addition, we did not find significant behavioral differences between pre- and post-artificial selection populations. However, I found that females from post-artificial selection populations had higher reproductive potential (more females dropped broods, and broods were larger for female size). This result is the opposite of what I predicted based on more rigorous selection, and is likely due to the additional food and better overall environment the fish experience during the more rigorous in-line breeding. My

results suggest that while the additional rigorous selection does not appear to influence invasive potential, the environment in which the lines are reared may be important when considering the invasive success of these pet trade fish.

Introduction

About one third of the world's worst aquatic invaders originate in the aquarium hobby industry (Padilla and Williams 2004). Through the aquarium trade, these species continue to be available retailers ranging in size from local hobby shop to national chain and can also be found online with relative ease (Patoka et al. 2016, Padilla and Williams 2004). Despite representing only 0.1% of known freshwater fish species, poeciliid species are responsible for about 10% of invasive freshwater fish list on the Global Invasive Species Database (Deacon and Magurran 2016). This may be in part due to ease of access – 'swordtails', platies, and fancy guppies are available for purchase online and at chain pet stores. It should be noted that almost all swordtails found within pet stores or online are actually *Xiphophorus helleri-maculatus* hybrids, and therefore many of the color variants found would not be seen in pure *X. helleri* fish. For example, the extent of melanin pigmentation, in some cases covering the total body including fins, cannot be found in *X. helleri*, as it does not carry the *Xmrk* gene responsible for low levels of melanic expression in the southern platyfish – it must be hybridized with *X. maculatus* to express this trait (Schartl and Meierjohann 2011). The bright coloration and interesting ornamentation, along with the relatively low maintenance, has led to these fish remaining popular for hobbyists and casual aquarium owners alike. Rixon et al. (2005) found varying strains of ‘swordtails’ in 85% of pet stores surrounding the Laurentian Great Lakes, the second most popular livebearing fish in their survey (following guppies, *Poecilia reticulata*). Unfortunately, livebearers have found their way outside of their native range and are responsible for the decline in aquatic invertebrates through aggressive foraging, as well as the decline of native fish species through faster population growth and/or competition (Deacon and Magurran 2016, Morgan et al. 2004, Englund 1999).

Release of livebearing fish to exotic locations has been both intentional and unintentional. Green swordtails, *Xiphophorus helleri*, were released on the Hawaiian Islands in the 1920s to control the mosquito population (Brock 1960). While there is no documentation on the origin of swordtails initially released (and whether or not they were hybrids), there have been numerous accidental and/or intentional release events in Hawaii since that time (pers. comm., Nico et al. 2018). Both *X. helleri* and the hybrids have been introduced to 31 countries worldwide, and the hybrids have also escaped or been cast-off from private aquariums and breeding farms (CABI 2018). As many released individuals originate in the pet or ornamental trade, many more of the invasive populations are likely hybrids than are actually documented as such (CABI 2018, Nico et al. 2018) The likelihood of a species becoming a successful invader is increased with continual release events, which contributes to the overall increased propagule pressure (i.e. both number of individuals released and number of discrete release events) of an exotic species (Blackburn et al. 2015, Simberloff 2009). Repeated release events not only increase propagule pressure but are also likely to increase the genetic variation in the exotic population, which may also increase chances of invasive success. While it is unlikely that intentional and unintentional releases can be totally stopped, and it is equally as unlikely that pet stores will stop stocking livebearing fish, it may be possible to involve the pet trade in a process of properly disposing of unwanted fish and reducing access to some of the ‘more invasive’ individuals.

Previously, I investigated whether the increased presence of melanin (responsible for black coloration) indicated individuals with ‘more invasive potential’ by comparing *X. helleri-maculatus* hybrid lines with no, minimal, and intense melanic coverage across their bodies. I found the most melanic line was more aggressive and bolder than the line without any melanic expression (“Wild Type”) but had significantly lower fecundity than both the Wild Type and the moderately melanic (“Spotted”) populations (see Chapter 3). No specific line had both high scores of the behaviors associated with invasive success and high fecundity, leading us to suggest that releasing a mix of phenotypes may be more detrimental than releasing any one phenotype on its own. Additionally, I hypothesized that the most melanic line’s (“Tuxedo”) reduced fecundity may have been due to the stronger form of artificial selection required to maintain the desired phenotype of that line (see Chapter 3), which reduces the influence of female mate choice.

In this study, I examine how the different breeding methods at the farms that provide fish to the pet stores could potentially influence invasive success, thereby leading to lines with increased invasive success. I examine two additional lines (Sky Blue and Brick Red) that experience an intense artificial selection process that is similar to the breeding process used to produce the Tuxedo line. Using the coloration of the lines to predict invasive success requires knowledge of genetic influence on the coloration, such that variation in coloration can be correlated with traits that are important for invasive success. While the melanic trait has a significant genetic component in *Xiphophorus* fishes (*Xmrk* gene, see Schartl and Meierjohann 2011, see also Walter and Kazianis 2001) and the melanocortin receptor genes (see Lampert et al. 2010, see also Ducrest et al. 2008) are known to play roles in various morphological, development, and behavioral traits in swordtail fish, much less is known about the relationship between development and behavior and the blue and orange pigmentation of the Sky Blue and Brick Red lines. Both carotenoids and pterins are responsible for orange or red pigmentation in the southern platy, *X. maculatus* (Basolo 2006). While carotenoids cannot be produced *de novo* and must be obtained via a fish's diet (Price et al. 2008, Tamaru et al. 2001), genetic control of pterins has been linked to the sex chromosomes in *X. maculatus* (Basolo 2006). The genetic control of red coloration when *X. maculatus* is hybridized with other *Xiphophorus* spp. is less clear, as results conflict on the location and/or number of alleles that may influence particular pigmentation patterns in the hybrids (reviewed by Basolo 2006). Much less is known about the iridescent coloration found in southern platyfish (including blue iridescence); there may be a heritable component to this particular coloration pattern, though Basolo (2006) also notes the possibility it is influenced by environmental and/or maternal effects.

All of the *X. helleri-maculatus* lines undergo weak artificial selection at some point in the process of breeding and sorting for the pet trade (Figure 4.1). Large numbers of fish are kept in outdoor breeding pools and are allowed to spawn freely for months at a time (pers. comm.; Tamaru et al. 2001). Fish are then harvested from these largescale breeding pools with baited traps and sorted for ‘sale grade’ coloration. This is a subjective process with no ‘hard and fast’ guideline, often leaving it up to those harvesting the fish to determine ‘desirable’ coloration after having culled any deformed, immature, or small fish (pers. comm., see also guidelines set out by Tamaru et al. 2001). However, some lines have traits that are more difficult to maintain than others (such as the intense melanic coverage of ‘Tuxedo’ fish or the presence of ‘blue’ coloration in the ‘Sky Blue’ line), and so fish collected from the largescale breeding pools are then isolated in small tanks or traps to encourage mating between specific pairs based on size and coloration, to further encourage propagation of the line (pers. comm., Tamaru et al. 2001). Therefore, these lines experience a much stronger form of artificial selection than fish in the Wild Type line (for example). This stronger form of artificial selection is characterized by strictly limited mating groups based on male color and size, and mate choice is limited as compared to the breeding pools.

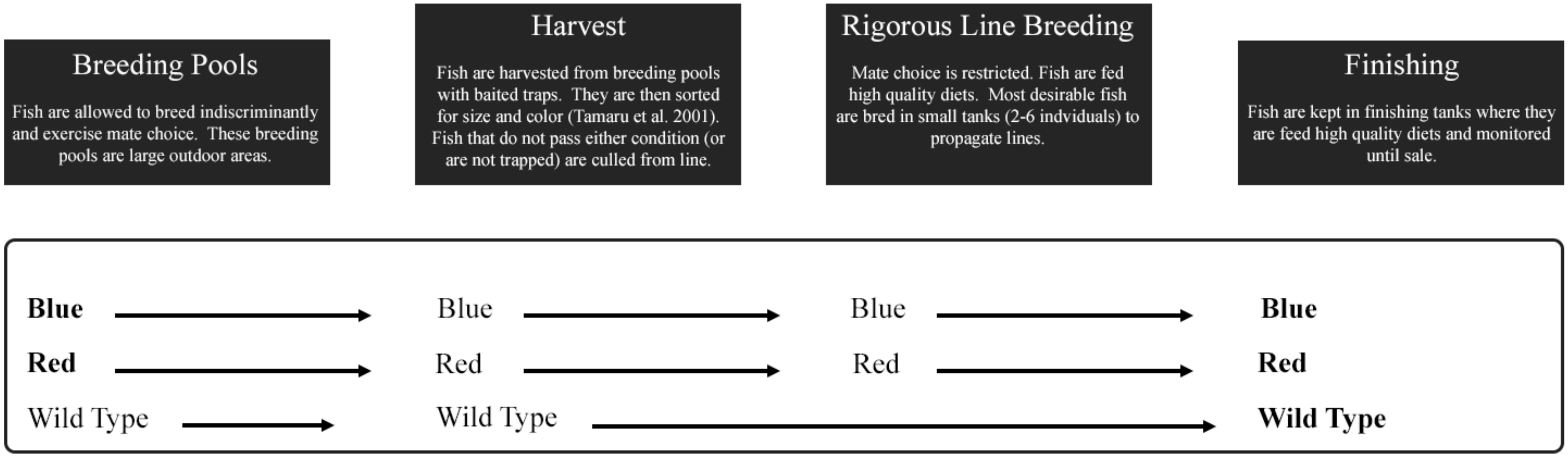


Figure 4.1. Linebreeding process and populations of *X. helleri-maculatus* sampled. The Wild Type fish do not undergo Rigorous Line Breeding. Fish were sampled from populations highlighted in bold, using baited traps for breeding pools and randomly selecting adults (50:50 males and females) from traps and finishing tanks.

In this study, I investigate whether this more rigorous form of linebreeding could influence the invasive potential by assessing three behaviors known to be associated with invasive success: exploration, aggression, and boldness (Chapple et al. 2012). These three behaviors have been documented in an invasive population of *X. helleri-maculatus* in a previous study (see Chapter 2). Additionally, I assess if this rigorous form of linebreeding changed variance within behaviors, as behavioral variation has been cited as potentially important in allowing invasive populations to overcome the many obstacles required to become established in an exotic location (reviewed by Chapple and Wong 2016). Finally, I assess if this rigorous linebreeding process influenced reproductive potential (as observed in the decreased fecundity of Tuxedo females observed in Chapter 3), as a species that cannot effectively reproduce cannot become established in an exotic location.

First, I will compare three lines at the ‘end’ of their stay on the farm, before being offered for sale to hobbyists or retailers (finishing tanks, see Figure 4.1). Two of these lines experienced rigorous (strong) selection, and the third line only experienced the weaker form of artificial selection. I expect that the lines that underwent more rigorous artificial selection process will have different behavioral medians, reduced behavioral variance, and lower reproductive potential as compared to the line that did not undergo the more rigorous artificial selection, as I observed similar results in a previous study in another line (Tuxedo) of swordtail hybrids that underwent a more rigorous artificial selection. Second, I will compare the two lines that experienced rigorous selection and examine the same three traits (median scores of invasive behaviors, variance in these behaviors, and reproductive potential) across individuals collected from the breeding pools *before* they had been sorted or put through the rigorous line-breeding process, and individuals from the end stage, *after* sorting and rigorous line-breeding. I expect that fish from the ‘Post-Selection’ populations will have higher behavioral medians and reduced behavioral variance when compared to the ‘Pre-Selection’ populations, as they have gone through this additional form of selection. However, as these fish are selected for size and breeding purposes, I expect that females from the Post-Selection populations will have larger broods than females from the Pre-Selection populations.

Methods

Study system

Xiphophorus helleri-maculatus hybrids (‘green swordtails’) are a subtropical, livebearing species of fish, popular in the pet trade. *X. helleri-maculatus* hybrids were collected directly from the aquarium trade for this study. All fish were acquired from Imperial Tropicals (Lakeland, Florida, USA). For the comparisons across lines, all fish were sampled directly from ‘finishing tanks’ as representation of fish would potentially move on to private individuals, and possibly release to a novel environment. Any sorting, breeding, or other forms of artificial selection have already occurred, and fish are held in ‘finishing tanks’ for quality control purposes that would be impossible in larger tanks or outdoor pools. These lines included the ‘Green Swordtail’ (henceforth referred to as ‘Wild Type’, N = 26), the ‘Sky Blue’ (henceforth referred to as ‘Blue’, N = 24), and the ‘Brick Red’ (henceforth referred to as ‘Red’, N = 22) lines. Because intense line-breeding is required to maintain the preferred coloration in the Blue and Red lines, but not the

Wild Type line (pers. comm., see Figure 4.2), these two lines experience more selection. The more rigorous selection process includes mating fewer individuals (2-6 fish) in smaller tanks and choosing to mate only the largest and most colorful males (pers. comm., see also Tamaru et al. 2001).

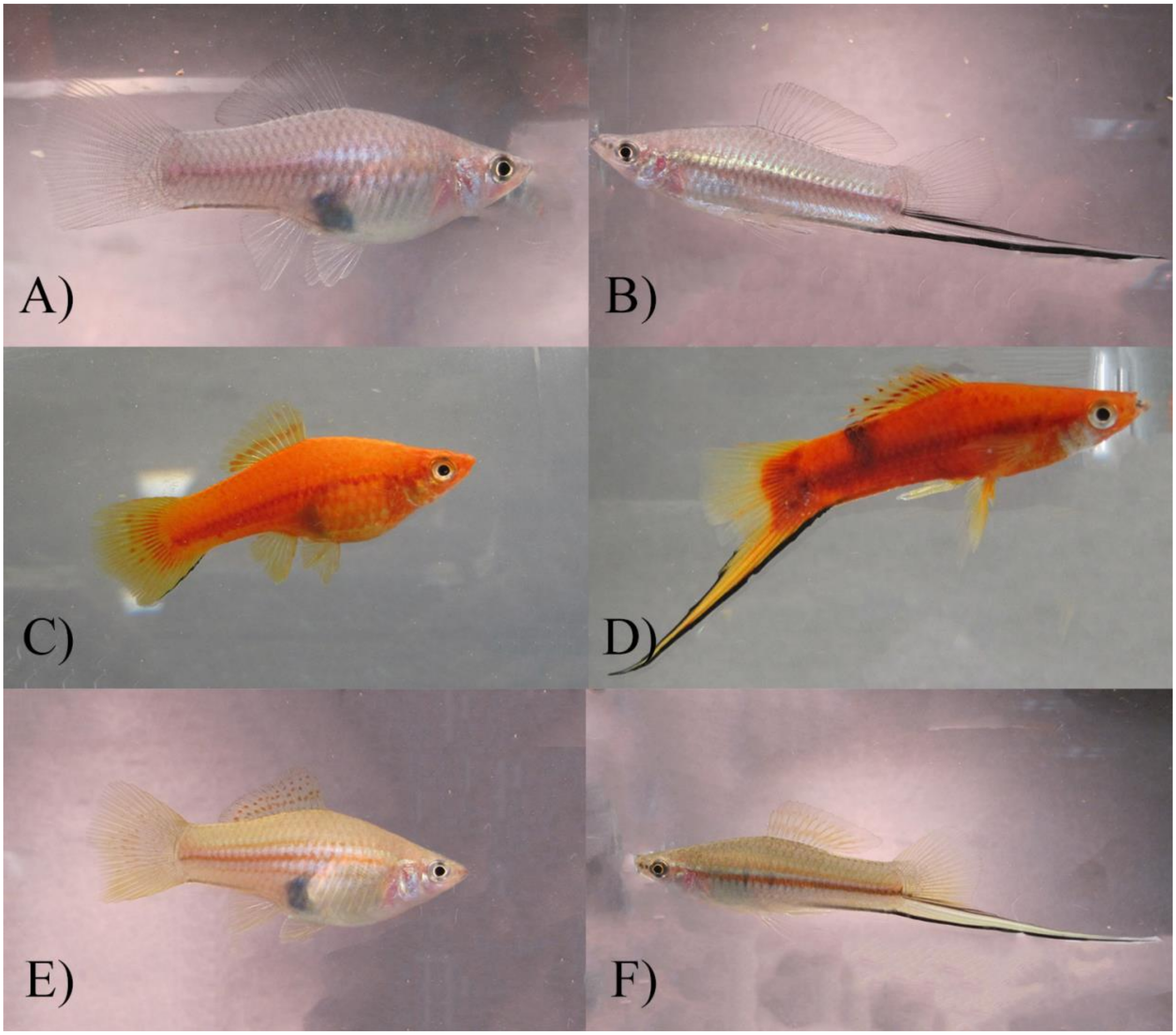


Figure 4.2 Three lines of *X. helleri-maculatus* hybrids were used in this study. Sky Blue (A-B, female and male respectively), referred to as “Blue”; Brick Red (C-D, female and male respectively), referred to as “Red”; and Green Swordtail (E-F, female and male respectively), referred to as “Wild Type”

Mimicking the harvesting method used by the fish farmers (baited traps), I also sampled individuals from the “Pre-selection” population (breeding pools) of both the Blue (N = 20) and Red (N = 21) lines. I selected adult fish, but chose fish randomly based on color and size. The fish from the breeding pools were collected so they could be compared to the “Post-selection” samples (finishing tanks) of the Blue (N = 24) and Red (N = 22) lines.

After collection, all fish were returned to a laboratory setting at Ohio University and kept in individual 5-gallon tanks. Fish were provided with refugia and kept on a 12 hour light-dark cycle. All fish were given at least two weeks to acclimate to the lab setting prior to any behavioral testing. Females that appeared pregnant were tested last, allowing them to drop their brood before any behavioral testing began. Females that did drop fry were moved to a new tank and given another two weeks to acclimate to new housing before testing.

To measure size, photos were taken in a small, glass V with a Canon PowerShot SD120015. Photos were measured using ImageJ 1.x (Schneider et al. 2012). Body area was measured for all fish on both sagittal planes, and then averaged for a fish’s score.

Behavioral testing

I tested fish for exploration of a novel environment, aggression towards a conspecific, and boldness under risk in that order to reduce possible carryover effect (see Bell 2013). During the exploration test, focal fish was placed in a novel 20-gallon tank divided into ten areas externally and monitored continuously for 300 seconds (s). Latency to begin movement, total number of movements, movements to different area, and total number of areas used were observed. Exploration testing was modeled after similar behavioral methods used to study three-spined sticklebacks (*Gasterosteus aculeatus*) by Bell and Stamps (2004), and has been used to study *X. helleri-maculatus* hybrids in a previous study (see Chapter 2). A mirror test was used to measure aggression towards a conspecific. Fish were kept in their home tank and were presented with a mirror. Latency until first approach and number of approaches were observed continuously for 300s. Methods have previously been used in another swordtail species, *X. multilineatus* (D'Amore et al. 2015, Chapter 1) and in *X. helleri-maculatus* hybrids (Chapter 2). During the boldness test, fish were allowed to fed undisturbed in their home tank for 30s before being 'surprised' with a net entering the tank. Fish were 'chased' for 15s before removal of net. If fish took shelter during this time, net 'hovered' near shelter for remaining time. Net was removed and then the latency to emerge from shelter and latency to resume feeding were measured for 300s post-netting as the indicator of boldness. Boldness methods were designed for use with *X. helleri-maculatus* hybrids in a previous study (see Chapter 2). Fish were given at least two hours between all tests to reduce potential of stress. All test were conducted within 48 hours to ensure fish remained in the same 'state'. Repeatability was previously found within *X. helleri-maculatus* hybrids (Chapter 2) and short-term repeatability was found to be an indicator of long-term repeatability in another swordtail species, *X. birchmanni* (Boulton et al. 2014).

Measuring reproductive potential

Adult females were isolated into individual tanks, which allowed us to assess whether females dropped fry, and to measure their brood sizes. Tanks were checked daily for fry. No female dropped more than one brood during study. Females that did drop fry were moved to a new tank and given another two weeks to acclimate to new housing before any testing began. All photos and size measurements were taken after females dropped fry. As fish arrived from the aquaculture farm pregnant, there was no control for the identity of the sire.

Comparisons across lines

Data were analyzed with program R v3.1.2 (R Development Core Team 2016). I compared all three behaviors and reproductive potential (number of females that dropped a brood, brood sizes) for the fish collected from the finishing tanks for all three lines. The Blue and Red lines represent the end point of the more rigorous form of artificial selection in the pet trade and the Wild Type line represents the end point of the less rigorous form of artificial selection. Data was checked for normality via Shapiro-Wilks testing. None of the data were normally distributed, which dictated the statistical analyses used. Behavioral measurements previously determined to be the most repeatable were selected to represent each of the three behaviors (see Chapter 2). Behavioral latencies were adjusted by subtracting individual’s score from highest possible score +1 to prevent 0 counts, and so high numerical scores were indicative of high behavioral expression.

Differences in behavior between sexes were analyzed with two-tailed Mann-Whitney U tests (Mann and Whitney 1947). I did not detect any significant difference in or exploration (W = 594.5, P = 0.662), aggression (W = 734, P = 0.264), or boldness (W = 529, P = 0.201) between the sexes, so the sexes were combined for further analysis. Differences in behaviors across populations were then used two-sized Mann-Whitney U tests to compare behaviors across populations. Differences in behavioral trait variance between populations were analyzed with two sample asymptotic tests using the ‘asympTest’ package (Coeurjolly et al. 2009), and also included a priori expectations based on expected differences in behavioral variance expected across the lines. Differences between behavior in females that reproduced and those that did not were also assessed with two-tailed Mann-Whitney U tests. Finally, differences in brood size while controlling for female size across lines was analyzed with Mann-Whitney U tests, using a one-sided design based on prior expectations.

Comparison within lines

To further assess whether the method of vigorous artificial selection (rigorous line-breeding) in some lines (in this case, Red and Blue) resulted in differences in behaviors or reproductive potential, I compared fish from the Red and Blue lines from the breeding pools (pre rigorous artificial selection) to fish from the same lines sampled from the finishing tanks (post rigorous artificial selection, see Figure 4.1). As the Wild Type line does not undergo this specific form of line-breeding, it was not included in this comparison. Neither median behavioral data nor behavioral variance were normally distributed. Therefore I compared median behavioral scores and behavioral variance with Mann-Whitney U tests and two sample asymptotic tests as explained above. I used one-sided Mann-Whitney U test to assess differences in behavior, behavioral variance, and brood sizes based on my prior expectations. Variation in whether a female had offspring and variation in number of offspring females produced were analyzed with generalized estimating equations (GEEs) using the ‘geepack’ package (Halekoh et al. 2006, Yan and Fine 2004, Yan 2002). Variation in whether a female had offspring or not was analyzed with a binomial model, including female’s line, when she was captured (Pre- or Post-Selection) and female’s overall size as covariates. Analyses started with a model that included all possible predictor variables and then found the minimal adequate model. The assessment was done longhand by removing predictor variables that did not significantly impact the model in a step-by-step process until I arrived at the minimum adequate model.

Results

Comparisons across lines

To determine if the more rigorous selection process used to maintain the Blue and Red lines led to changes in the three behaviors associated with invasive success, I compared these two lines to the Wild Type line where selection was weaker with Mann-Whitney U tests. There were no significant differences between lines in boldness, aggression, or exploration (Table 4.1). Additionally, there were no significant differences in variance of these behaviors between the three populations, with one exception (Table

4.2, Figure 4.3). The Red line has significantly less variance in exploration than the Wild Type line.

Table 4.1

Ordered comparisons of behavioral scores across comparisons of lines that underwent both rigorous and less rigorous forms of artificial selection

A. Boldness	W	P
Blue vs. Red	297.5	0.460
Blue vs. Wild Type	389	0.128
Red vs. Wild Type	297.5	0.460

B. Aggression	W	P
Blue vs. Red	215	0.273
Blue vs. Wild Type	297	0.770
Red vs. Wild Type	215	0.274

C. Exploration	W	P
Blue vs. Red	343	0.083
Blue vs. Wild Type	397	0.096
Red vs. Wild Type	343	0.083

Wald = Wald test statistic; significant P values in bold

Table 4.2

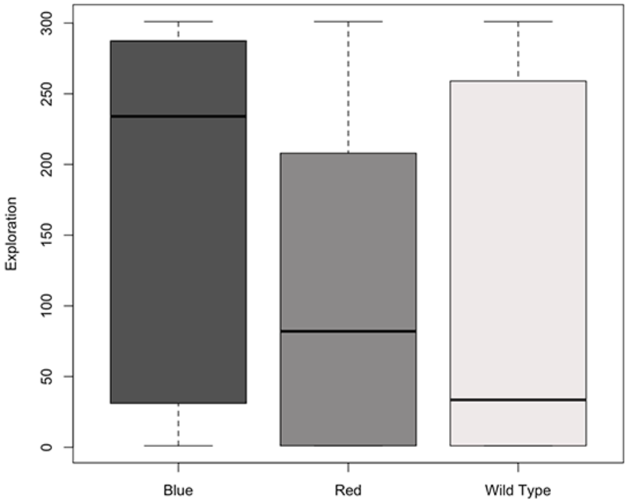
Asymptotic test comparison of difference in variance across lines that underwent both rigorous and less rigorous forms of artificial selection

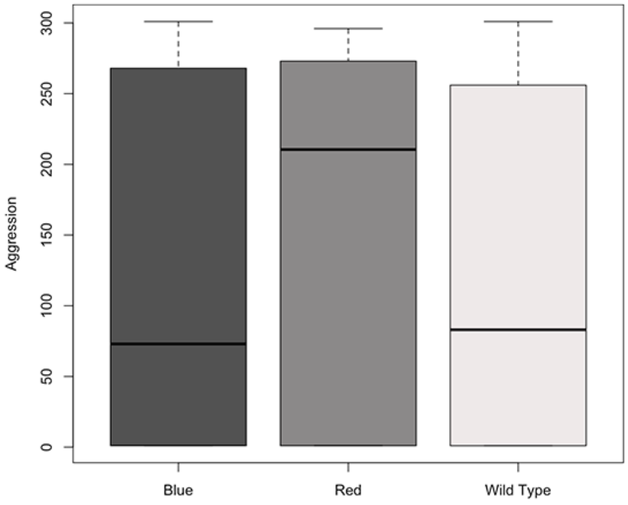
A. Boldness	θ	P
Blue vs. Red	0.585	0.599
Blue vs. Wild Type	1.301	0.903
Red vs. Wild Type	0.813	0.792

B. Aggression	θ	P
Blue vs. Red	0.607	0.545
Blue vs. Wild Type	-0.110	0.456
Red vs. Wild Type	-0.749	0.277

C. Exploration	θ	P
Blue vs. Red	1.925	0.054
Blue vs. Wild Type	-0.210	0.417
Red vs. Wild Type	-2.053	**0.020**

θ = value of unified θ statistic; significant P values in bold


Exploration
0
50
100
150
200
250
300
Blue
Red
Wild Type


Aggression
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Wild Type

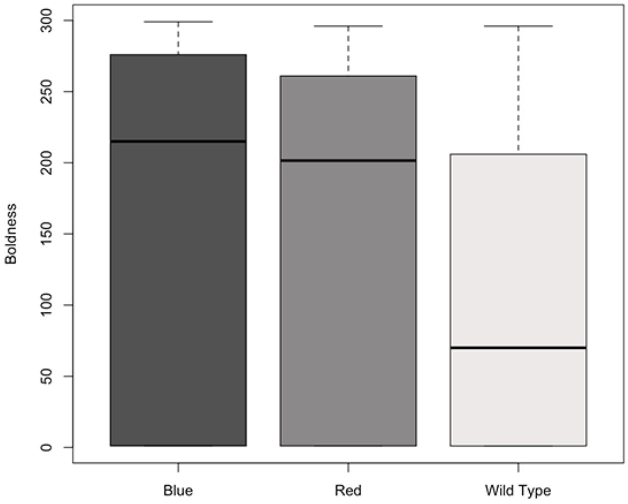
Boldness
0
50
100
150
200
250
300
Blue
Red
Wild Type

Figure 4.3. Variation in behaviors across the three lines of *X. helleri-maculatus* collected from the end stage of the aquarium trade process (finishing tanks). Boxes: 25th and 75th percentiles; center line: median; 1.5 times the interquartile range of the data

I did not observe a difference in median exploration (W = 172, P = 0.453), aggression (W = 288.5, P = 0.427), or boldness (W = 86.5, P = 0.720) scores when comparing females who dropped a brood (N = 20) to females who did not (N = 20) during the study period. I did not observe a significant correlation between a female's brood size (controlled for her overall body size) with exploration (r_s = 0.383, N = 20, P = 0.095), aggression (r_s = -0.259, N = 20, P = 0.271), or boldness (r_s = 0.030, N = 20, P = 0.902). Additionally, reproductive females from the Red line had significantly less offspring than females from either the Wild Type or Blue lines (Table 4.3, Figure 4.4).

Table 4.3

Ordered comparisons of brood sizes across different lines of *X. helleri-maculatus* females

Line	W	P
Blue vs. Wild Type	25	0.733
Red vs. Wild Type	9	**0.027**
Blue vs. Red	40	**0.005**

Significant P values in bold

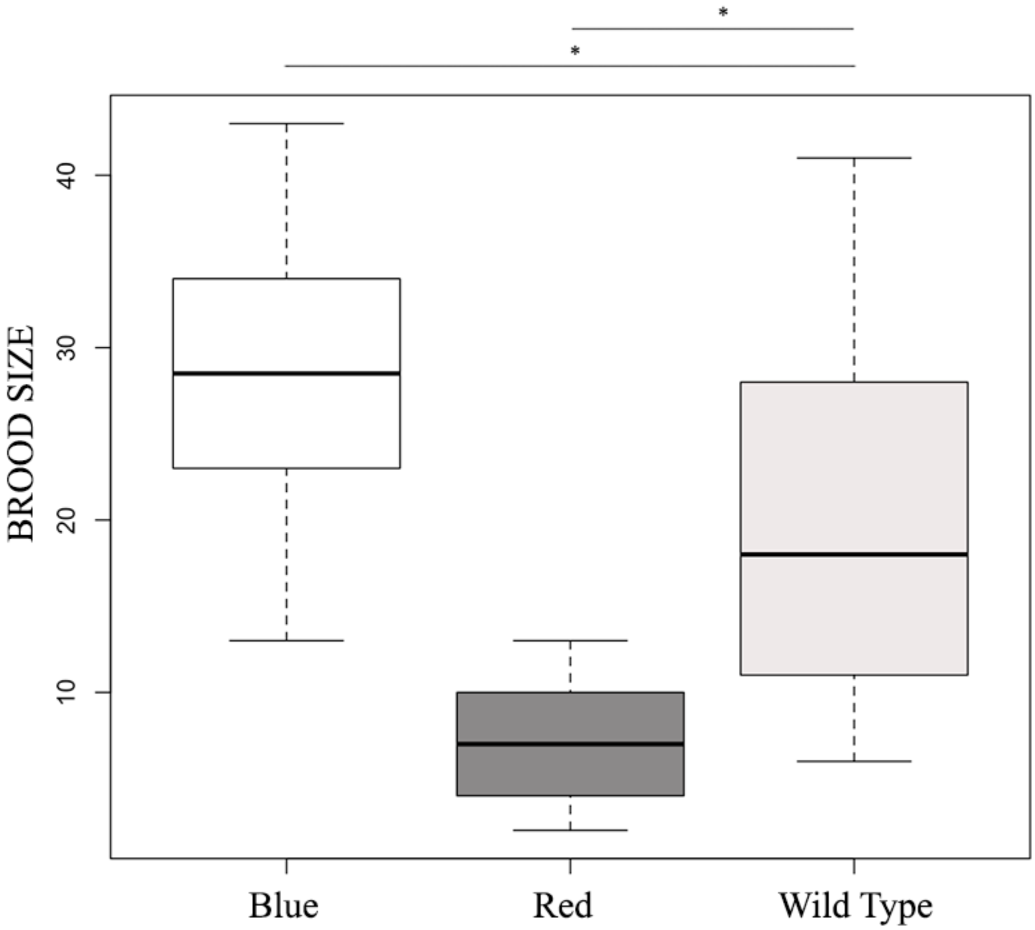

Figure 4.4. Median brood size in reproductive females in Blue (more rigorous selection), Red (more rigorous selection), and Wild Type (less rigorous selection) from the finishing tanks. Boxes: 25th and 75th percentiles; center line: median; 1.5 times the interquartile range of the data

Comparisons within lines

To further examine the possibility that the method of selective breeding and sorting (stronger selection) could influence the behaviors of the fish that enter the pet trade, I compared fish sampled from the breeding pools prior to sorting (“Pre-Selection”) to those that had been selectively bred and sorted (“Post-selection”, see Figure 4.1). As there were no differences between the Blue and Red lines in any of behaviors or behavioral variance (see Results for comparisons across lines), I combined data from the Blue and Red lines for these analyses, giving us a Pre-Selection sample size of 41, and Post-Selection sample size of 46.

There was no significant difference in mean exploration scores between males and females (W = 0.865, P = 0.523). However, I did detect significant differences between the sexes in both aggression (W = 667.5, P = 0.019) and boldness (W = 1183, P =0.034). Males were significantly more aggressive than females (Figure 4.5), while females were significantly bolder than males (Figure 4.6). Therefore, I split the Pre-Selection (female = 22, male = 19) and Post-Selection populations (female = 25, male = 21) by sex in the analyses of aggression and boldness.

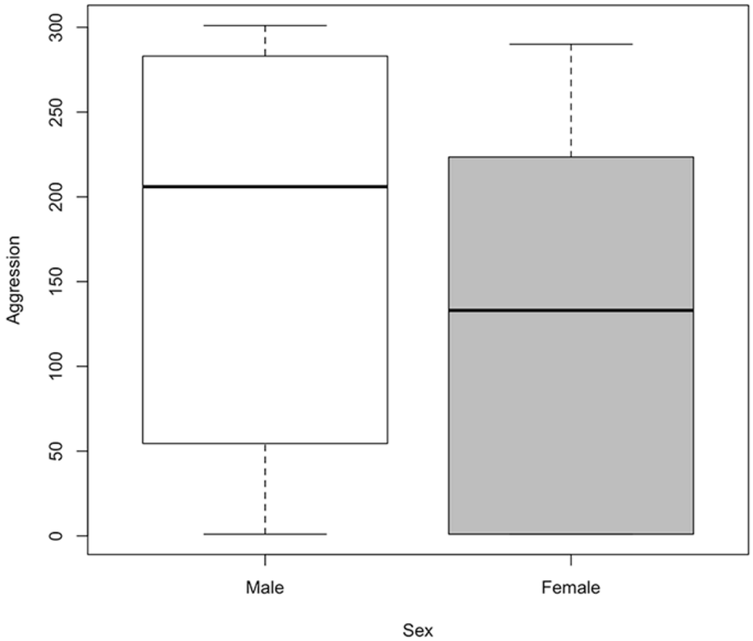

Figure 4.5. Difference in aggression between males and females (combined Pre- and Post-Selection populations). Higher aggression score = attacked mirror image more quickly. Boxes: 25th and 75th percentiles; center line: median; 1.5 times the interquartile range of the data

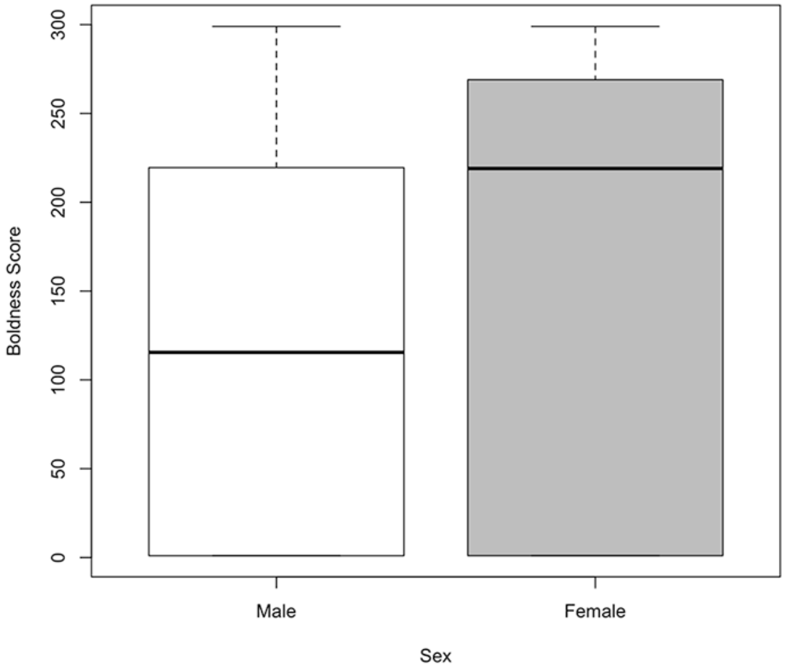

Figure 4.6. Difference in boldness in males and females (combined Pre- and Post-Selection populations). Higher boldness score = faster recovery time after simulated predator attack. Boxes: 25th and 75th percentiles; center line: median; 1.5 times the interquartile range of the data

There were no significant differences in exploration (W = 861, P = 0.760) before or after rigorous selection processes. I did not detect differences in aggression between Pre- and Post-selection populations, nor did I detect significant differences in boldness between Pre- and Post-selection populations, regardless of sex (Table 4.4) In addition, I detected no significant differences in the variance of exploration (θ = -0.417, P = 0.388) or in the behaviors divided by sex (Table 4.5) when comparing the Pre-Selection and Post-Selection populations.

Table 4.4.

Ordered comparisons of behavioral scores across populations that have undergone varying degrees of artificial selection

	Females		**Males**	
	W	P	W	P
Aggression	298.5	0.309	326	0.137
Boldness	161.5	0.853	233.5	0.171

Table 4.5

Asymptotic test comparison of difference in behavioral variance across populations that have undergone varying degrees of artificial selection

	Females		**Males**	
	θ	P	θ	P
Aggression	2.812	0.998	2.234	0.987
Boldness	0.295	0.616	0.753	0.744

θ = value of unified θ statistic

Finally, I examined factors influencing reproductive potential. The GEE was performed as a binomial model and assessed whether or not a female dropped a brood. The best fit model explaining variation in whether or not a female dropped was the null model, which meant none of the observed factors played a significant role in the probability of dropping a brood. A one-sided Mann-Whitney U test revealed that females from the Post-selection population had significantly larger broods than those from the Pre-selection population after controlling for brood size (Figure 4.7).

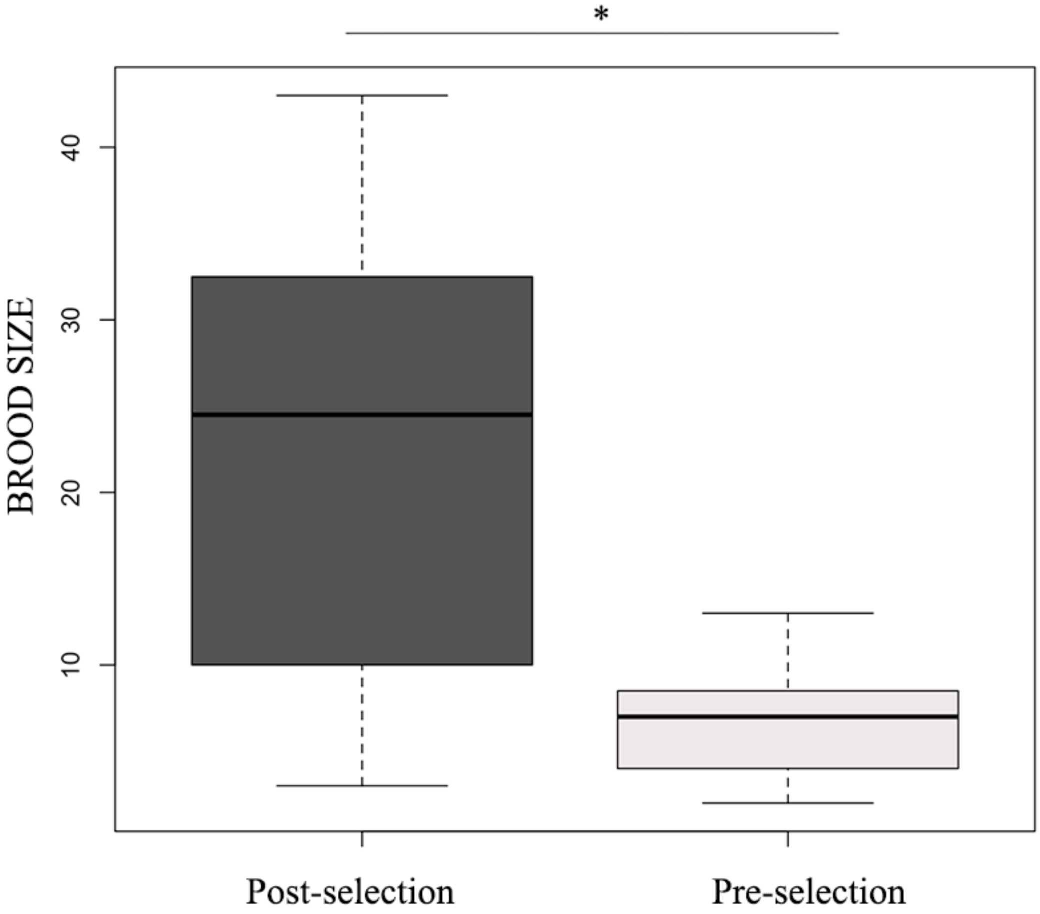

Figure 4.7. Difference in brood sizes between females from Pre- (from the Breeding Pools prior to artificial selection) and Post-Selection (from the Finishing Tanks, post all artificial selection) populations. Boxes: 25th and 75th percentiles; center line: median; 1.5 times the interquartile range of the data

Discussion

The method of additional sorting and breeding that is needed to produce the desired traits in some lines of the *X. helleri-maculatus* hybrids does not appear to influence the behaviors of these lines. I did not detect differences in the observed behaviors across lines that varied in their method of selection (Blue and Red as compared to Wild Type). In addition, I detected no differences when I compared the behaviors of the fish prior to sorting and breeding ("Pre-Selection", captured from Breeding Pools) to the fish after rigorous artificial selection ("Post-Selection", captured from Finishing Tanks) in the two lines that receive additional selection. These results were surprising given that I predicted the methods of sorting and selective breeding could have increased behavioral traits correlated to the morphological traits under selection, and potentially decrease genetic variation in both the morphological traits and the behaviors. As for reproductive potential, I did not detect differences in the numbers of females that dropped broods or brood size that I could attributed to the methods of selection in our comparison across lines. I did, however, detect differences between the lines in reproductive potential, with the Red line having significantly smaller brood sizes for female size. I also detected differences in brood l within lines between the breeding pools and finishing tanks (pre- and post-rigorous selection), that could potentially suggest that methods of selecting fish from the breeding pool, and/or more rigorous selection puts more pregnant females that produce larger broods into the pet trade. However, these results were in the opposite direction of what I predicted rigorous selection would produce. I discuss the potential reasons for these results as well as their significance for reducing the impact of the release of these fish from the pet trade below.

There are two possible reasons for why I did not see increased behavioral scores or decreased variance in the lines with increased artificial selection pressures as I predicted. First, our predictions were based on the assumption that the behaviors I examined were correlated with the traits that were being selected. Previously, I detected behavioral differences between three lines of *X. helleri-maculatus* hybrids that had been bred for varying levels of melanin expression (see Chapter 3). I found that the most melanic line was bolder than the other lines, and both lines displaying melanin were more aggressive than the line without melanin (Wild Type line). The presence of melanin has a strong genetic influence in *Xiphophorus* fishes and specifically *X. helleri-maculatus* hybrids (Schartl and Meierjohann 2011, Walter and Kazianis 2001), and relationships between behaviors (such as aggression) and melanin have been observed across vertebrate taxa (see review by Ducrest et al. 2008). The traits under selection in this study included vibrancy of the blue coloration, red coloration, and male size. While it is possible that neither the red nor blue coloration are genetically correlated with aggression or boldness in this species, I suggest that it is more likely that environmental influences swamp out the genetic variation within the colors, given that diet can play such an important role in the red/orange coloration (Price 2008, Tamaru et al. 2001).

A second possible reason for the lack of differences in behaviors due to the more rigorous selection is that the method of collecting the fish (bait trapping) prior to the selective breeding and sorting may have already reduced the variation that would have been available for selection to act on. Baited traps is the only method by which fish are removed from the breeding pools, and this collection method has been shown to select for bolder individuals across taxa (see Biro 2013, Biro and Dingemanse 2009). Given that boldness and aggression are often correlated in swordtails (see D’Amore et al. 2015, Chapter 1; Chapter 2), trapping from the breeding pools may have already reduced behavioral phenotypes found within the populations prior to the more rigorous selection. While this hypothesis is worth testing, it would need to be tested in another setting where it would be possible to compare both fish that enter traps and those that that do not, as the breeding pools at this site were too deep for seining or electroshocking.

Both the Blue and Wild Type lines had higher reproductive potential than the Red line (Table 4.3, Figure 4.3), suggesting that the Red line may be less successful invaders than either of the other two lines. This is an important trait that should be compared across lines. Populations that produce more offspring in a novel environment will have faster population growth rates, and faster growth rates can lead to higher probability of spread than populations with slower growth rates (Caswell et al. 2003). Increasing the number of propagules introduced to an environment through reproduction could be likened to the theory of propagule pressure, which suggests the chances of success of establishing invasive populations in exotic locations is increased when the number of individuals released is also increased (Blackburn et al. 2015, Simberloff 2009).

While it is difficult to know exactly how influential reproduction is and exactly in what way it influences invasive success (Sol and Maspons 2016), it is clearly an important trait to consider. Demographic models have implied that another important consideration is repeated introduction events, which increase the likelihood a species will become established in an exotic environment (Cassey et al. 2018, Lockwood et al. 2005, Cassey et al. 2004). This issue is exacerbated by the fact that livebearing fish store sperm, and therefore even without males, females can give birth to multiple broods after release. Individual, pregnant female Trinidadian guppies (*Poecilia reticulata*), another species of livebearing fish, were able to establish successful, long-term populations in a mesocosm experiment with an 86% success rate (Deacon et al. 2011). I found that the number of females that dropped broods in the laboratory (I.E. were pregnant at the time of collection) was greater for the fish collected from the finishing tanks as compared to the breeding pools (Figure 4.7), and there were differences in brood sizes between the pre- and post-selection females. Therefore, our results suggest the process of rigorous line-breeding used to maintain lines such as ‘Sky Blue’ may also increase the number of pregnant females that enter the pet trade as well as the number of offspring each female produces for her size. The higher probability of females being pregnant could be to the small mating blocks used to encourage ‘desirable’ females to mate with ‘desirable’ males, potentially forcing females to mate that would otherwise not mate. However, as the fish that leave the fish farm will still have to spend time in a pet store prior to being sold to the public, at least for fish that are released by pet owners, the effect of fewer pregnant females prior to rigorous selection is likely to be lost. The larger brood sizes in the finishing tanks are more likely due to environmental influences, as fish in these tanks have higher access to a more food, as compared to the fish in the breeding pools, where there is much more competition for a more limited supply of food.

I detected significant differences in the behaviors between males and females in the Red and Blue lines that would be interesting to examine further. Males were more aggressive than females, which was not too surprising given the differences in reproductive strategies between the sexes in the *Xiphophorus* system (Morris et al. 1995, Morris et al. 1992, Franck and Ribowski 1987). Females, on the other hand, were bolder than males. All the fish in the study were captured by bait trapping from the breeding pools (either for this study directly, or by the farm prior to selective breeding process), a method which has been shown to select for bolder fish in other systems, as well as bolder animals across taxa (Biro 2013, Biro and Dingemanse 2008). This difference between males and females in boldness could suggest that trapping influences males and females differently, in that while both bold and shy males can be trapped, only the bolder females are trapped. It is also important to note that the ‘Pre-’ and ‘Post-Selection’ populations do not come from the same generation; the ‘Post-Selection’ fish in the ‘finishing tanks’ were collected from the breeding pools the year before, and between years the breeding pools are drained and restarted. In addition, the two groups of fish may have experienced different rearing environments in the breeding pools due to temporal changes between years. This study may be worth repeating when fish from the same generation are sampled and tested ‘Pre-Selection’, and then again sampled after the fish farm has put the generation through all the stages of the rigorous breeding process to see if the same patterns are observed.

Finally, it would be worth further exploring additional factors that could influence the potential invasive success of the different lines. In relation to reproductive potential and invasive behaviors, our results suggest that the Blue line might be the most problematic. The Blue line had the highest reproductive potential, and while not significant, the highest mean scores for boldness and exploration. Other factors that should be included in comparing invasive success across lines in the future, at least for locations with potential predators, is conspicuousness to predators. Conspicuousness to predators will depend not only on the coloration of the fish, but on environment. Both ambient light conditions and background coloration will significantly influence the conspicuousness of the Blue as compared to Red lines, and thus their ultimate success at invading localities with predators may depend on specific aspects of the environment.

In conclusion, I did not find differences in behaviors or behavioral variation between lines representing both rigorous and not-as-rigorous artificial selection, nor did I find differences when comparing behaviors and behavioral variations of the rigorously selected lines pre- and post-sorting and breeding. I did find differences in brood sizes between lines at the final stage, but it was not associated with how the lines were selected, However, differences in proportion of females that dropped broods and brood sizes were detected between the Pre- and Post-selection populations, suggesting the environment in the finishing tanks may increase reproductive potential. I suggest that it will be important to further consider the environments in which the fish are reared in addition to additional differences between the lines in working to reduce behaviors associated with invasive success in this popular aquarium and widespread pest species.

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