

*UNCORRECTED PROOF*

## **Boldness and predator evasion in naturally hybridizing swordtails (Teleostei: *Xiphophorus*)**

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**Abstract** Correlations among behavioral traits can generate trade-offs and constrain phenotypic evolution. Interspecific hybridization has the potential to alter behavioral trait correlations, but the effect of hybridization on suites of behavioral traits has received relatively little attention. We evaluate how natural hybridization changes the relationship between boldness (time of emergence and proportion of time out of shelter) and response to a simulated predator threat in swordtails (Teleostei: *Xiphophorus*). In poeciliid fishes, bold individuals have increased survival in the presence of predators. This non-intuitive observation may arise as a result of bold individuals being more likely to engage in anti-predator behaviors. Contrary to our prediction, bold individuals were less likely to perform a fast-start response to a predator threat. This correlation was consistent among populations and species but was only significant in hybrids. The observed correlation between boldness and anti-predator behavior could impact hybrid fitness and the evolvability of hybrid lineages. More generally, our findings suggest that hybridization could influence the integration of behavioral phenotypes, as has been amply documented for morphology. Animal personality and behavioral syndromes could therefore play an important role in the evolutionary fate of natural hybrids [*Current Zoology* 61 () : – , 2015 ].

**Keywords** Trait compensation, Fast-Start, Anti-predator behavior, hybridization, Behavioral syndrome, Animal personality

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Phenotypic integration, or correlations among functionally-related traits, has profound consequences for organismal fitness. For example, correlations among traits may generate phenotypic trade-offs where beneficial traits covary with detrimental traits (Ballentine, 2006; Ghalambor et al., 2004; Sih et al., 2004a; Sih et al., 2004b) or the co-expression of traits where one may compensate for other, less optimal traits (DeWitt et al., 1999; Mikolajewski and Johansson, 2004; Oufiero and Garland, 2007). If phenotypic correlations have a genetic basis, heritable covariation between traits places limits on the rate and direction of evolution (Arnold, 1992; Cheverud, 1984; Smith and Rausher, 2008). Among morphological traits, numerous studies have shown that hybridization can rapidly and dramatically alter phenotypic integration in ways that affect the fitness of hybrids relative to parental populations (Albertson and Kocher, 2005; Grant and Grant, 1996; Murren et al., 2002; Seehausen, 2004). Specifically, hybridization can relax phenotypic trade-offs and genetic constraints by reducing trait correlations (Parsons et al., 2011; Seehausen, 2004).

Just as with morphology, behavioral traits can also exhibit phenotypic integration. Correlated suites of functionally-related traits are termed personalities or behavioral syndromes, and have received considerable attention in recent years (Réale et al., 2007; Sih et al., 2004a; Sih et al., 2004b). There has been surprisingly little work, however, on how natural hybridization may change phenotypic integration among behavioral traits. Here we explore the influence of hybridization on the relationship between two functionally-related behavioral traits, boldness and predator evasion, in natural populations of swordtail fish (Poeciliidae: *Xiphophorus*).

Boldness, or the propensity of individuals to engage in risky behavior (Brown et al., 2005; Wilson, 1998; Wilson et al., 1994) is a trait with important and often conflicting implications for fitness. Boldness can be beneficial in the acquisition of mates (Godin and Dugatkin, 1996; Reaney and Backwell, 2007) and food (Stamps, 2007; Ward et al., 2004) but can expose individuals to greater predation risk (Sih et al., 2004a; Sih et al., 2004b; Stamps, 2007). While studies have shown that boldness can decrease survivorship (Bremner-Harrison et al., 2004; Dugatkin, 1992; Wilson et al., 1993) there are numerous cases where bolder individuals are *more* likely to survive in the presence of predators (Fuiman et al., 2010; Godin and Davis, 1995; Réale and Festa-Bianchet, 2003; Smith and Blumstein, 2010). In poeciliid fishes, most studies have shown that bolder fish have higher survivorship (Brown et al., 2005; Godin and Davis, 1995; Harris et al., 2010; Smith and Blumstein, 2010; but see Dugatkin, 1992).

The variable fitness consequences of boldness may reflect trait compensation, whereby the costs of expressing certain trait values can be mitigated by other traits (Mikolajewski and Johansson, 2004; Oufiero and Garland, 2007). Thus, the counterintuitive finding that boldness increases survival with predators may arise from bold behavior being compensated for by anti-predator behaviors (Smith and Blumstein, 2010). In fish, the primary evasive action in response to a predator attack is so-called “fast-start” behavior, characterized by the fish rapidly bending into a “C” shape then explosively extending the caudal peduncle, propelling the individual forward rapidly (Domenici and Blake, 1997). Fast-start increases the likelihood of surviving attacks by fish (Langerhans, 2009; Walker et al., 2005) and bird (Katzir and Camhi, 1993) predators. If boldness is compensated for by anti-predator behaviors, we should expect to observe positive correlations between measures of boldness and the probability of performing a fast-start.

We evaluated the relationship between boldness and evasive responses to predators in a naturally hybridizing complex of the northern swordtails *Xiphophorus malinche* and *X. birchmanni* (Culumber et al., 2011; Rosenthal et al., 2003). *Xiphophorus malinche* inhabits small, shallow headwater streams at high elevations, while *X. birchmanni* is found in larger rivers at low elevation and hybrids are found at intermediate elevations. *Xiphophorus birchmanni* show repeatable within-individual correlations in traits characteristic of a latent boldness axis (Boulton et al., 2014). Hybrids backcross readily to both parental species (Culumber et al., 2014), but hybrid zone structure is maintained by superior fitness of *X. malinche* and *X. birchmanni* at upper and lower extremes, respectively (Culumber et al., 2012), and by selection against hybrids at numerous incompatibility loci across the genome (Schumer et al., 2014).

Our goals were twofold. First, we sought to determine whether, among individuals, boldness covaries with fast-start behavior. We predicted that boldness would be positively correlated with the tendency to respond (fast-start) to a simulated predator attack. Second, we evaluated the impact of hybridization on the relationship between boldness and fast-start behaviors at both the individual and population levels.

## 1 Materials and Methods

### 1.1 Fish collection and behavioral trials

Sexually mature male and female *Xiphophorus* were collected during May and June 2007 using baited funnel traps, from six sites: two *X. birchmanni*: Garces (Latitude, Longitude; 20.940°, -98.282°) and Huitznopala (20.910, -98.425); two *X. malinche*: Chicayotla (20.925°, -98.577°) and Malila (20.734, -98.715); and two hybrid: Tlatemaco (21.023°, -98.790°) and Calnali-mid (20.894, -98.610). All locations were previously identified as either parental or hybrid using 3 autosomal and 1 mitochondrial molecular markers (Culumber et al., 2011). Fish were temporarily housed in aerated coolers and transported to the Centro de Investigaciones Científicas de las Huastecas “Aguazarca” (CICHAZ) in Calnali, Hidalgo, Mexico. Fish were housed, by population, in 76-liter aquaria on a natural light/dark cycle, for at least 48 hours before trials began. Additionally, field notes from underwater observation and electroshocking during other field visits provide information on the fish communities in these three represent areas along the elevational gradient, including detection/non-detection of putative predators (Table 1). Since fish collections have been made as far back as 2003 (Culumber et al., 2011), rarity and lack of detection of predatory fish species at intermediate and high elevation sites, respectively, provides strong evidence that predatory fish do not commonly co-occur with hybrids nor *X. malinche*.

To quantify boldness, we used standardized trials to measure time to emergence from shelter and proportion of time out of shelter, both of which correlated strongly with other boldness measures in a previous study (Boulton et al., 2014). Fish were placed individually into a circular plastic tank (57 cm top diameter × 47 cm bottom diameter × 25 cm depth) filled with water to a depth of 15 cm. At the center of the tank, four small rocks provided cover. Natural behavior of *Xiphophorus* from our sample sites is to seek cover in the substrate, and all fish took cover in the rocks immediately upon release. In preliminary trials, fish remained in shelter for the duration of the trial if placed in isolation in the tank. In the wild and in

captivity, *Xiphophorus* shoal with other swordtails (Buckingham et al., 2007; Wong and Rosenthal, 2005). We therefore placed five juvenile *Xiphophorus* (standard length 10–15 mm) in an 8 liter plastic aquarium at the opposite end of the tank from the rocks to provide visual shoaling cues. We measured two indices of boldness, latency to emerge from shelter (Brown et al., 2005; Cote et al., 2010; Harris et al., 2010; Wilson and Godin, 2009) and proportion of time spent out of cover (Harcourt et al., 2010). If a fish did not emerge within 10 minutes the trial was ended and the fish was assigned a latency to emerge of 600 seconds. Latency to emerge was log transformed (Brown et al., 2005) and proportion of time out was arcsine square root transformed (Harcourt et al., 2009). We used principal components analysis (PCA) on both measures to create a combined index of boldness. While the two measures of boldness were correlated ( $r = -0.64$ ,  $P \leq 0.0001$ ) PCA could obscure differences between taxa if the relationship between both measures of boldness differed among taxa. We found that the correlation between our two measures of boldness did not differ among species-type (*X. malinche*:  $r = -0.67$ ,  $P \leq 0.0001$ ; *X. birchmanni*:  $r = -0.55$ ,  $P \leq 0.0001$ ; hybrids:  $r = -0.63$ ,  $P \leq 0.0001$ ; Fisher z-transform: all pairwise comparisons  $P > 0.05$ ); it is therefore unlikely that PCA obscured differences among taxa. The first principal component explained 82% of the variance in subject behavior. Loadings indicated that high PC1 scores were associated with greater proportion of time out and faster latency to emerge.

Immediately after boldness trials, we used a simulated predator attack to measure the propensity to perform a fast-start. Each subject was placed in a separate plastic tank adjacent and identical to that used for the boldness trials, but entirely bare, and given 5 minutes to acclimatize. A cardboard model of an Amazon kingfisher *Chloroceryle amazona* was then released down a monofilament line stretched taut over the test tank (bird drop length = 325 cm, bird model height = 284 cm, bird model drop angle = 30°). Fish responses were recorded with a Panasonic PVGS59 digital camcorder at 30 frames per second. We recorded whether or not the fish responded to the stimulus by initiating a fast-start. We operationally scored a fish as performing a fast-start if, during the period the bird model passed over the test pool, the fish brought its snout toward its caudal peduncle (forming a “C” shape, Domenici and Blake, 1997). All other behaviors, including swimming backward slowly ( $n = 4$ ), cessation of swimming ( $n = 29$ ) and no apparent change in swimming behavior ( $n = 71$ ), were operationally scored as “no response”.

## 1.2 Statistical analysis

We used logistic regression to estimate the effect of taxon (hybrid, *X. birchmanni* or *X. malinche*), population nested in taxon, and sex on whether or not an individual performed a fast-start. Differences between taxa were evaluated using odds ratio tests.

Analysis of variance (ANOVA) was used to investigate the effects of taxon, population nested in taxon, sex, and fast-start (yes or no) on boldness (PC 1). Differences between taxa were evaluated using Tukey’s HSD post-hoc tests.

To evaluate if the relationship between fast-start and boldness differed among taxa, we took two approaches. First, to evaluate behavioral integration we calculated point-biserial correlation coefficients (for hybrids and parentals, respectively) which are appropriate for estimating correlations between continuous variables (e.g. boldness) and dichotomous variables

(e.g. fast-start; Glass and Hopkins, 1996). Second, to evaluate the dependence of fast-start behavior on boldness, we performed logistic regressions for parentals and hybrids where fast-start was dependent on boldness PC1.

## 2 Results

Logistic regression indicated that taxa (*X. birchmanni*, *X. malinche*, and hybrids) and populations within taxa both varied in their likelihood to perform a fast-start ( $\chi^2_{\text{taxon}} = 16.8$ ,  $df = 2$ ,  $P = 0.0002$ , population nested in taxon,  $\chi^2_{\text{pop[taxon]}} = 25.09$ ,  $df = 3$ ,  $P \leq 0.0001$ ). *Xiphophorus birchmanni* performed fast-starts more often (48.5% of trials,  $n = 66$ ; Fig. 1), hybrids fast-started slightly less often (38.3% of trials,  $n = 47$ ) and *X. malinche* were least likely to fast-start (16.2% of trials,  $n = 74$ ). The odds of a fast-start in *X. birchmanni* were equivalent to that of hybrids (odds ratio = 1.39,  $P = 0.46$ ). By contrast, the odds of a fast-start in *X. malinche* were significantly different than in *X. birchmanni* (odds ratio = 7.08,  $P \leq 0.0001$ ) and hybrids (odds ratio = 5.11,  $P = 0.005$ ). There was no sex difference in propensity to fast-start ( $\chi^2_{\text{sex}} = 2.67$ ,  $df = 1$ ,  $P = 0.1024$ ). ANOVA indicated that taxa ( $F_{2,179} = 8.34$ ,  $P = 0.0003$ ), and population nested in taxon ( $F_{3,179} = 3.63$ ,  $df = 3$ ,  $P = 0.014$ ) differed in boldness. *Xiphophorus birchmanni* were boldest (least-square mean = 0.44,  $SE = 0.16$ ) and significantly bolder than both *X. malinche* (Tukey's HSD:  $P = 0.001$ ) and hybrids (Tukey's HSD:  $P = 0.002$ ). *Xiphophorus malinche* were shyest (least-square mean = -0.34,  $SE = 0.15$ ) and did not differ from hybrids (least-square mean = -0.40,  $SE = 0.18$ ) with respect to boldness (Tukey's HSD:  $P = 0.96$ ). The relationship between boldness and fast-start was weak ( $F_{1,179} = 3.76$ ,  $df = 1$ ,  $P = 0.054$ ) but consistent among taxa with bolder fish being less likely to fast-start (Fig. 1). Males and females did not differ in boldness ( $F_{1,179} = 0.1$ ,  $df = 1$ ,  $P = 0.3194$ ).

Point-biserial correlation coefficients indicated that the relationship between fast-start and boldness was significant only in hybrids ( $r = -0.31$ ,  $n = 47$ ,  $P = 0.036$ ) but not in *X. birchmanni* ( $r = -0.16$ ,  $n = 66$ ,  $P = 0.19$ ) nor *X. malinche* ( $r = -0.05$ ,  $n = 74$ ,  $P = 0.67$ ). This pattern was also found with logistic regression; hybrids showed a significant negative relationship between boldness and fast-start ( $b = 0.55$ ,  $SE = 0.27$ ,  $\chi^2 = 4.08$ ,  $P = 0.04$ ) and parentals did not (*X. birchmanni*:  $b = 0.29$ ,  $SE = 0.22$ ,  $\chi^2 = 1.68$ ,  $P = 0.1943$ ; *X. malinche*:  $b = 0.11$ ,  $SE = 0.25$ ,  $\chi^2 = 0.18$ ,  $P = 0.67$ ).

## 3 Discussion

Our results do not support the hypothesis that bold animals minimize predation costs as a result of increased likelihood to display anti-predator behaviors (Fuiman et al., 2010). We predicted that boldness would covary positively with the tendency to perform a common anti-predator behavior, a fast-start response (Katzir and Camhi, 1993; Langerhans, 2009; Walker et al., 2005). Surprisingly, we found a significant correlation in the opposite direction where bolder individuals were less likely to fast-start in hybrids (Fig. 1A, B). This negative correlation could put hybrids at a greater risk to predators, representing a potential source of selection against hybrids. However, further work is necessary to determine whether hybrids are captured at a higher rate due to this negative correlation. If the observed phenotypic correlation in hybrids is a result of a genetic correlation between boldness and likelihood to fast-start, it may result in genetic constraint limiting the

evolvability of adaptive phenotypes with respect to anti-predator responses (Dochtermann, 2011). Additionally, in terms of boldness and fast-start behaviors at the taxa-level, *X. birchmanni* were significantly bolder and significantly more likely to perform a fast-start than *X. malinche*. This difference between parental species indicates the influence of genetic, environmental, epigenetic factors such as maternal effects or a combination.

Neither parental species demonstrated a correlation between boldness and fast-start behaviors within individuals. However, as a species overall, *X. birchmanni* were both bolder and more likely to fast-start than either hybrids or *X. malinche*. It has been observed in other poeciliids that populations sympatric with fish predators are bolder than those without or with fewer predators (Brown et al., 2005; Godin and Davis, 1995; Harris et al., 2010; Smith and Blumstein, 2010). *X. birchmanni* are co-distributed with a greater number of piscivorous bird (CONABIO 2008) and fish predators (Table 1; Miller et al., 2005). Predatory birds, such as the kingfisher, are in general more common at lower elevations (Martínez-Morales et al., 2007; Remsen, 1991; Skutch, 1957) and three of four species of kingfisher in the region co-occur with only *X. birchmanni* and hybrids, but not *X. malinche* (Martínez-Morales et al., 2007; CONABIO, 2008; eBIRD, 2014). Piscivorous fishes are absent from the upper stream reaches where *X. malinche* occur and rarely at intermediate elevations, but several are present in lowland sites in sympatry with *X. birchmanni* (Table 1). Greater predator richness and abundance at low elevations suggests probable increased experience with predators for *X. birchmanni*. Our data demonstrating that *X. birchmanni* were bolder and more likely to fast-start in response to a simulated attack than hybrids or *X. malinche* are consistent with these other poeciliid studies demonstrating that boldness covaries with predator occurrence. However, it is not clear whether such differences between species reflect genetic or plastic (i.e., based on experience) differences.

Certain environmental factors such as water depth may influence the susceptibility to or experience with aerial predator attacks. Though *X. birchmanni* and *X. malinche* occur at opposite ends of an elevation gradient, they inhabit similar pools. *X. malinche* occur in shallow stream pools (Rauchenberger et al., 1990) at only six headwater sites (Culumber et al., 2011). As expected along an elevation gradient, streams increase in order and become wider and deeper main channels where *X. birchmanni* occurs. However, *X. birchmanni* inhabit shallower, slower moving pools along the edges of the main stream and in backwaters (Fisher and Rosenthal, 2010). These two species therefore are found in comparable pool habitats of similar depths. These small pools may limit predation from fish predators (Rypel et al., 2007) such as *Gobiomorus dormitor* which occurs in flowing main channels and is broadly co-distributed with *X. birchmanni* (Miller et al., 2005). These shallow pools may, however, put fish at greater risk from bird predators, such as kingfishers (Vilches et al., 2013). Thus, given the decreasing distribution of avian predators as a function of increasing elevation, *X. malinche* likely experience less predation and therefore, have less need to engage in fast-start compared with *X. birchmanni*. Furthermore, the similarity in pool types used by the two species (Rauchenberger et al., 1990; Fisher and Rosenthal, 2010) suggests that differences in bird predation pressure would likely not be due to differences in water depth. We nonetheless cannot entirely rule out that differences in behavior were a function of absolute differences in water depth between species at the exact time of or immediately prior to fish collection.

It is important to note that our study does not rule out the possibility that boldness exhibits plasticity related to other environmental inputs that might vary along the elevation gradient. Riesch et al. (2009) found that wild-caught mollies *Poecilia mexicana* from different habitat types differed in boldness, but behavioral differences disappeared when fish were reared in a common garden. This suggests that in some species boldness behaviors may be largely plastic. A study of a separate poeciliid, *Brachyrhaphis episcopi*, found significant heritability of boldness traits of lab-bred individuals from low- and high-predation populations (Brown et al., 2007). Their study also found a significant influence of experience on boldness in the laboratory. Differences in boldness between *X. birchmanni* and *X. malinche* may be due to heritable variation, plasticity based on experience with predators, or a combination. Alternatively, it has recently been shown that escape behaviors resulting from phenotypic integration in lizards (Lancaster et al. 2010) and exploratory boldness behavior in zebrafish (Wisenden et al., 2011) are sensitive to maternal effects. Effects of maternal size and age on offspring size have recently been demonstrated in *X. birchmanni* (Kindsvater et al., 2012), but the extent to which maternal effects influence boldness behaviors is not yet known. We cannot rule out such effects with our data set. However, such maternal effects would nonetheless need to be at a taxa level given the differences in boldness and propensity to fast-start between species, but lack of correlation of behaviors within individuals. Since there was no correlation between traits within *X. birchmanni* nor *X. malinche* it would also suggest separate maternal effects for boldness and fast-start. Future work including a controlled breeding design could dissect the potential environmental and maternal components of behavioral syndromes in *Xiphophorus*. Ultimately, the mechanism underlying species-level differences does not change the observation that only hybrids exhibited a significant, negative correlation between boldness behaviors at the level of individual. This result in hybrids highlights the ability of natural hybridization to influence behavioral syndromes with potential survival consequences. Furthermore, since hybrids represent genetic mosaics of two species from different environments with potentially different predation pressure and were the only group to show a significant correlation between traits, this may suggest a genetic component to these behaviors.

Natural hybrids have often been considered to be effective evolutionary sinks, when their fitness is lower than that of parental species (Barton and Hewitt 1985). In the natural hybrid zones of *Xiphophorus birchmanni* and *X. malinche*, hybrids have generally been found to be equally or more fit than parental species in terms of physiological performance (Culumber et al., 2012) and mating success (Culumber et al., 2014). However, the negative correlation between boldness and fast-start may indicate a mechanism by which hybrids are at a disadvantage, if the lack of fast-start behavior in bold fish results in higher mortality from avian predators. Natural hybrids have provided numerous insights into the nature and consequences of phenotypic integration among multiple morphological traits. While numerous studies have characterized behavioral traits in hybrids, e. g. mate choice (Fisher et al., 2006; Grant and Grant, 1997; Hatfield and Schluter, 1996), signal production (Grant and Grant, 1997; Qvarnstrom et al., 2006), anti-predator behaviors (Fitzpatrick, 2008), there has been little work on how hybridization affects correlated suites of behaviors. Furthermore, despite a growing body of literature suggesting that common underlying mechanisms should affect multiple behaviors in a range of contexts (O'Connell and Hofmann, 2012) we know relatively little about the genetic architecture and functional trade-offs associated with suites of behavioral traits (Dochtermann and Roff, 2010). Given the potential for hybridization to alter genetic architecture (Guillaume and Whitlock,

2007), and given the promise of genetic mapping studies in non-model organisms in light of next-generation sequencing technology, hybrids may prove useful in studying the evolutionary genetics of multivariate behavioral phenotypes.

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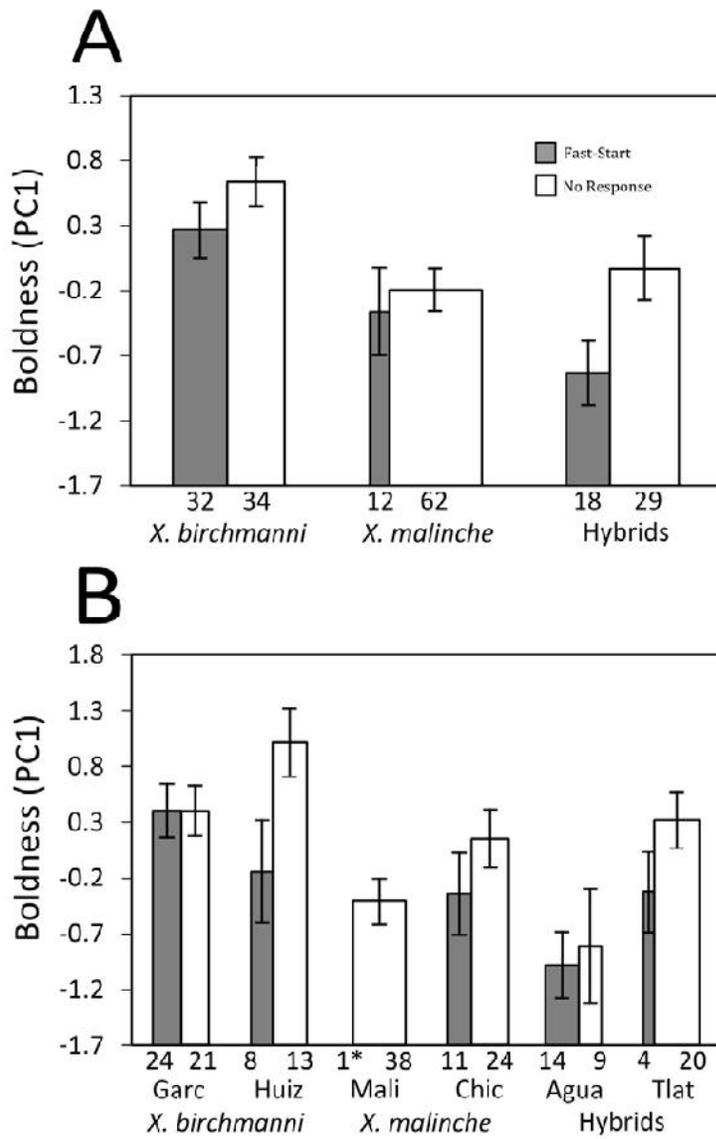
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**Table 1** Representative fish communities present in sympatry with each of the focal groups from the study

| Focal Species        | Fish species present                      |
|----------------------|---|
| <i>X. birchmanni</i> | <i>Amatitlania nigrofasciata</i>          |
|                      | <i>Astyanax mexicanus</i> <sup>PR</sup>   |
|                      | <i>Cyprinella lutrensis</i>               |
|                      | <i>Gambusia flexipennis</i>               |
|                      | <i>Gambusia regani</i>                    |
|                      | <i>Gobiomorus dormitor</i> <sup>PR</sup>  |
|                      | <i>Herichthys carpintes</i> <sup>PR</sup> |
|                      | <i>H. pantostictus</i> <sup>PR</sup>      |
|                      | <i>Ictalurus punctatus</i> <sup>PR</sup>  |
|                      | <i>Notropis tropicus</i>                  |
|                      | <i>Oreochromis sp.</i>                    |
|                      | <i>Poecilia mexicana</i>                  |
|                      | <i>Poeciliopsis gracilis</i>              |
|                      | <i>Pseudoxiphophorus jonesii</i>          |
| <i>X. variatus</i>   |   |
| hybrids              | <i>Astyanax mexicanus</i> <sup>PR</sup>   |
|                      | <i>H. pantostictus</i> <sup>PR</sup>      |
|                      | <i>P. jonesii</i>                         |
|                      | <i>Tampichthys ipni</i>                   |
|                      | <i>X. variatus</i>                        |
| <i>X. malinche</i>   | <i>P. jonesii</i>                         |

Typical of gradient streams, species richness and the presence of predatory species increases at lower elevations where *X. birchmanni* occur. Species with known tendencies as fish predators (Miller et al. 2005) are indicated with a superscript PR.



**Fig. 1 Mean boldness (PC1,  $\pm SE$ ) and performance of fast-starts in simulated predator strikes for taxa (A) and populations (B)**

Width of bars indicates proportion of individuals who performed a fast-start. Asterisk indicates insufficient data to calculate mean. Sample sizes per group are indicated below each bar.

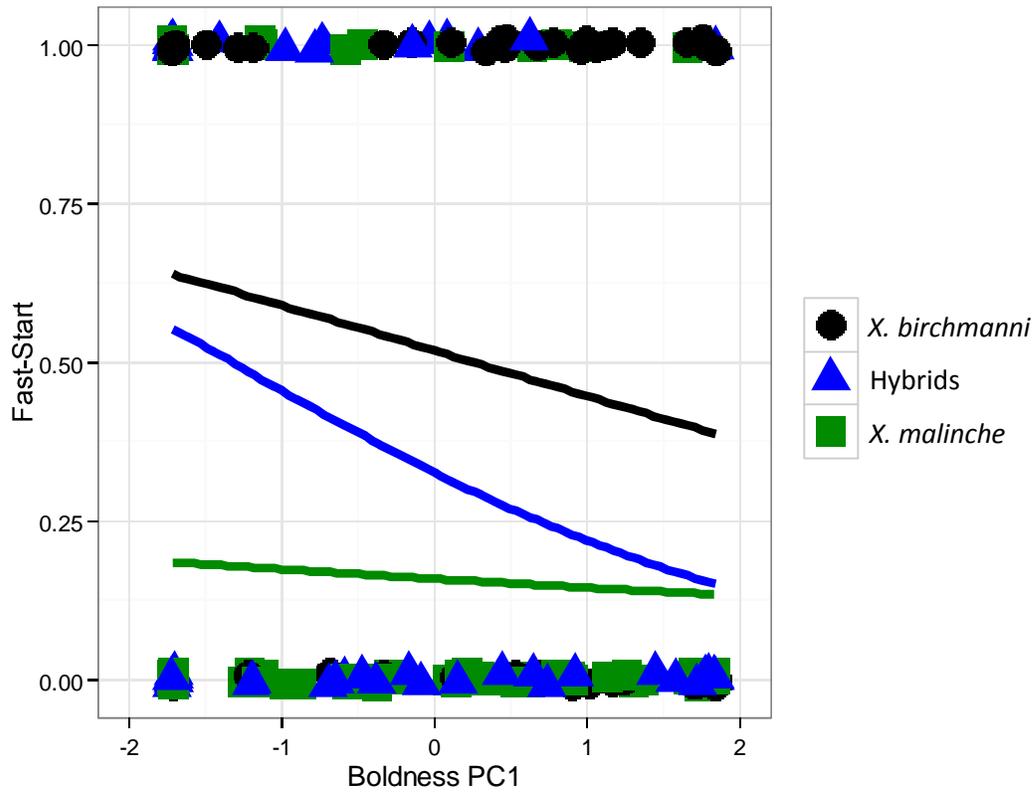


Fig. 2 Logistic regression curves displaying the relationship between boldness (PC1) and fast-start probability for *X. birchmanni*, *X. malinche* and hybrids