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A cancer-causing gene is positively correlated with male aggression in *Xiphophorus cortezi*

Dr. André A. Fernandez
Department of Carcinogenesis, The University of Texas – M. D. Anderson Cancer Center; Science Park/Research Division; 1808 Park Road 1C; Smithville, Texas 78957, Phone: (512) 237-9457; Fax: (512) 237-2437

André A. Fernandez: aafernandez@mdanderson.org

Abstract

The persistence of seemingly maladaptive genes in organisms challenges evolutionary biological thought. In *Xiphophorus* fishes, certain melanin patterns form malignant melanomas due to a cancer-causing gene (*Xiphophorus* melanoma receptor kinase; *Xmrk*), which arose several millions years ago from unequal meiotic recombination. *Xiphophorus* melanomas are male biased and induced by androgens however male behavior and *Xmrk* genotype has not been investigated. This study found that male *X. cortezi* with the spotted caudal (Sc) pattern, from which melanomas originate, displayed increased aggression in mirror image trials. Furthermore, *Xmrk* males (regardless of Sc phenotype) bit and performed more agonistic displays than *Xmrk* deficient males. Male aggressive response decreased when males viewed their Sc image as compared to their non-Sc image. Collectively, these results indicate that *Xmrk* males experience a competitive advantage over wild-type males and that intrasexual selection could be an important component in the evolutionary maintenance of this oncogene within *Xiphophorus*.

Keywords

sexual selection; *Xiphophorus*; *Xmrk*; melanoma; aggression; behavior; genetic linkage

Introduction

Initial investigations of cancer focused on the proximate causes of the disease (*e.g.*, progression, susceptibility); however, more recent studies have addressed the ultimate causes behind the persistence of cancer (for discussion see Greaves, 2007). This more evolutionary approach has led to our understanding that the maintenance of genes with oncogenetic potential can result from factors such as genomic conflict (‘selfish’ genes: Summers *et al.*, 2002; Kleene, 2005), antagonistic coevolution (maternal-fetal interactions: Lala *et al.*, 2002; Summers & Crespi, 2005), antagonistic pleiotropy (Summers & Crespi, 2008), and sexual selection (Fernandez & Morris, 2008); establishing evolutionary trade-offs as a central theme underlying the evolutionary biology of cancer (Graham, 1992; Greaves, 2000; Crespi & Summers, 2006). For example, the tumor suppression gene *TP53* is not only effective at suppressing cancer, but also stem cells that replenish worn out tissues (*i.e.*, antagonistic pleiotropy; for review Rodier *et al.*, 2007). Thus, expression of *TP53* represents a trade-off because it functions to decrease tumor susceptibility but also accelerates senescence (Tyner *et al.*, 2002; Weinstein & Ciszek, 2002; Campisi, 2005; Krtolica, 2005).

Cancer-causing genes (oncogenes) that are encoded on the sex chromosomes pass directly from parent to offspring through the germ line. Such oncogenes can persist across many

generations and therefore should be influenced by selection. In the *Xiphophorus* melanoma model, a germline oncogene (*Xiphophorus* melanoma receptor kinase, *Xmrk*) that originated from a tandem gene duplication of the *Xiphophorus* epidermal growth factor receptor (*Egfr*) gene has remained functional despite being deleterious (Schartl *et al.*, 1995; Weis & Schartl, 1998) and located in an extremely unstable genomic region (Volff *et al.*, 2003). The event that led to the creation of *Xmrk* is believed to predate the divergence of *Xiphophorus* fishes (*i.e.*, platyfishes and swordtails) with *Xmrk* being subsequently lost in numerous species (Kazianis & Borowsky, 1995; Weis & Schartl, 1998). *Xmrk* is the predominant gene underlying disease progression and melanomas do not occur if this gene is disrupted (Schartl *et al.*, 1999). Although the initial discovery of *Xiphophorus* melanoma susceptibility was induced through hybrid crosses (Gordon, 1927; Kosswig, 1928), it is now clear that *Xiphophorus* fishes are susceptible to spontaneous melanomas in the absence of hybridization (Kallman, 1971; Borowsky, 1973; Fernandez & Bowser, 2008). In both hybrid and non-hybrid melanomas, the pathway leading to transformed phenotype results from the overexpression of the *Xmrk* oncogene within the Ras/Raf/MAPK signaling cascade with subsequent tumors originating from species-specific macromelanophore patterns (Schartl *et al.*, 1995; Meierjohann & Schartl, 2006). Such malignancies are costly due to the invasion of underlying muscle tissue, which ultimately impairs swimming ability (Schartl *et al.*, 1995;Fernandez & Bowser, 2008). Given this, explaining how the *Xmrk* oncogene has been passed down through the germline for million of years has remained a considerable challenge for evolutionary biologists (Meierjohann & Schartl, 2006).

In addition to being a model organism in which to study cancer, work on *Xiphophorus* fishes has been instrumental in developing several concepts of sexual selection. These include the evolution of female mate choice (Basolo, 1990, 1995), genetic determinants of size at sexual maturity and sex determination (Kallman *et al.*, 1973), as well as alternative reproductive strategies (Ryan & Causey, 1989). *Xiphophorus* (Poeciliidae, Cyprinodontiformes) is a morphologically diverse group of fishes that uses visual cues to not only compete for but also select potential mates (body size: Ryan *et al.*, 1990; Morris *et al.*, 1992; Fernandez *et al.*, 2008; sword size: Basolo, 1990; Benson & Basolo, 2006). Several behavioral studies have highlighted the specific importance of melanin patterns in sexual selection (Morris, 1998; Basolo & Trainor, 2002; Morris *et al.*, 2003; Moretz & Morris, 2006) and found that the same phenotype can play a role in both mate choice and male-male competition. For example, the vertical body bars in numerous species are attractive to females and males can vary the expression of their vertical bars to deter rival males during agonistic encounters (Morris *et al.*, 1995). However, the vast majority of *Xiphophorus* behavioral studies have focused on melanin patterns comprised of micromelanophores, which are smaller (up to 100 μM) and relatively more evenly spaced pigment cells than the larger (300–500 μM) overlapping macromelanophores that can be associated with the *Xmrk* oncogene (Weis & Schartl, 1998). This dearth of investigation is surprising given that the preservation of *Xmrk* has only occurred in those *Xiphophorus* that possess macromelanophore patterns (M patterns) suggesting these M patterns may play a key role in the evolutionary maintenance of *Xmrk* (Meierjohann & Schartl, 2006; Fernandez & Morris, 2008).

Recently, positive selection was demonstrated for the *Xmrk* oncogene in *Xiphophorus cortezi* providing the first empirical evidence that sexual selection plays a role in the continued maintenance of this deleterious gene. Fernandez and Morris (2008) found that females prefer males with the spotted caudal (Sc) M pattern that is associated with the presence of the *Xmrk* genotype to wildtype males without the Sc phenotype (and *Xmrk* genotype). In *X. cortezi*, melanomas are male biased and their incidence is greatest in males 9–12 months old (Schartl *et al.*, 1995) and, therefore, *Xmrk* has the potential to substantially decrease the reproductive lifespan of males (Fernandez & Morris, 2008). Whether or not the increased attractiveness of Sc patterned males or the possible employment of different

mating strategies (*e.g.*, terminal effort signaling; Polak & Starmer, 1998; Hunt *et al.*, 2004) compensates for the potentially reduced reproductive lifespan of *Xmrk* males remains to be elucidated. Similarly, male aggression and the *Xmrk* oncogene has not been explored in *Xiphophorus* despite the fact that Schartl and colleagues (1995) state anecdotally that melanomas in *X. cortezi* occur most frequently in 'sexually active males of high social rank'. This suggests that there may be a relationship between dominance hierarchy (*i.e.*, male-male competition) and the *Xmrk* oncogene.

The primary goal of this study was to determine if male aggression in the Northern swordtail *X. cortezi* is correlated with the presence of the Sc M pattern or the oncogene *Xmrk*. *Xiphophorus cortezi* is polymorphic for the *Xmrk* oncogene, which is located on the X and/or Y chromosomes. *Xmrk* is an essential component for the phenotypic expression of Sc (Schartl *et al.*, 1995; Weis & Schartl, 1998). Unlike *Xmrk*, the precise genomic location of *Sc* is not known although it is hypothesized to be autosomally determined based upon breeding experiments (Kallman, 1971). Sc is an extremely asymmetrical pattern that typically consists of one or more irregular elongations that commence at the base of the caudal fin and extend roughly one-third of the length of the caudal fin (Fig. 1; Kallman, 1971; Schartl *et al.*, 1995). All individuals with the Sc pattern have *Xmrk* (Schartl *et al.*, 1995; Weis & Schartl, 1998); however, this phenotype has incomplete penetrance (Kallman, 1971). Therefore, it is feasible that an individual who lacks phenotypic expression of Sc but has the *Sc* genotype can possess the associated *Xmrk* genotype. This study will use molecular biology techniques and male mirror image stimulation tests to specifically address the following questions: 1) Can individuals without the phenotypic expression of Sc possess the *Xmrk* genotype? 2) Are males with the naturally occurring Sc pattern more aggressive than wild type males who lack the Sc phenotype but have it artificially applied? 3) Are males with the *Xmrk* genotype more aggressive than wild type males without the *Xmrk* genotype? and 4) Does male aggressive response differ based upon Sc phenotype?

Methods

Specimen collection and housing

Xiphophorus cortezi males used in this study were collected from five natural populations within the Rio Panuco basin: Arroyo Tanute N 21 39 123, W 99 02 127; Arroyo Chalpuhuacanita N 21 12 364, W 98 40 153; Rio San Martin N 21 22 173, W 98 39 543; Arroyo Tecolutlo N 21 07 270, W 98 28 075; and Arroyo Conchita N 21 33 5, W 98 59 320 (Hidalgo and San Luis Potosi provinces; Mexico). All males were collected as adults during two field seasons: December 2005 (Mean ± σ, Conchita: SL= 39.3 ± 3.78 mm, n = 30; Tanute: SL = 33.87 ± 4.71 mm, n = 15) and April 2006 (Mean ± σ, Chalpuhuacanita: SL = 38.7 ± 3.9 mm, n = 23; San Martin: SL = 38.08 ± 3.81 mm, n = 26; Tecolutlo: SL = 39.76 ± 3.02 mm, n = 13). Standard length (SL) was defined as the distance from the tip of the snout to the base of the caudal peduncle. In the laboratory, males from each population were housed in communal tanks with females from the same locality. All fish were maintained under standard laboratory conditions throughout the experiment consisting of 12L:12D cycle, daily feeding (Tetramin® flakes), and a constant temperature of 22° C (±1° C).

DNA isolation, PCR, and *Xmrk* genotyping

Males were anesthetized with tricane methanesulphonate (MS-222) and fin clipped after their last MIS test (see below) and DNA was extracted using DNeasy® tissue kit (Qiagen Inc.) following the manufacturer's instructions. Total elution volume was 100 μl. The presence of *Xmrk* was determined by cross-referencing the polymerase chain reaction (PCR) products of two newly developed primers sets. These primers were designed from published *Xiphophorus montezumae* sequences in GenBank (Accession #s AY298857, AY298858).

The published sequences in Genbank are derived from *Xmrk* specific clones (Volff *et al.*, 2003), however there are regions of these sequences that are shared by both the *Xmrk* oncogene and the proto-oncogene (*Efgr ortholog*). The following primer set was used to screen for the presence of the *Xmrk* genotype: "Montoncoup" sense primer 5′-GGGTCATAAATCACTCATCCATC located in the promoter region at nt 21–43 (nt numbering according to AY298858; Volff *et al.*, 2003) and "Dwnmont2" antisense primer 5′-ACAAGTTTGTGGAAATAAACCTGAACTC located in Intron 1 at nt 688–715 (nt numbering according to AY298858; Volff *et al.*, 2003). Because the Montoncoup primer corresponds to a region that is specific to the *Xmrk* oncogene, this primer set amplifies a single ~ 700 bp fragment if the individual male has the *Xmrk* oncogene (*Xmrk* deficient = no band). For the amplification of oncogene and proto-oncogene products, the following primers were developed: "Montoncoup5" sense primer 5′-GATGTTACTTTAGTTCTGGAGTC located at nt 2956–2978 (nt numbering according to AY298857; Volff *et al.*, 2003) and "Montoncodwn1" the antisense primer 5′-TCAGTTTGTTGGATCAGAGATG located at nt 266–287 (nt numbering according to AY298858; Volff *et al.*, 2003). The Montoncoup5 primer corresponds to a sequence found in both the oncogene and protooncogene, therefore the second primer set (Montoncoup5/ Montoncodwn1) produces bands for both the proto-oncogene and the oncogene. The use of this second primer set enabled 1) verification of the presence of amplifiable DNA 2) validation of the findings of the first PCR screening (*i.e.*, Montoncoup/Dwnmont2). The final concentration of the primers was 100 nM.

The total reaction volume of all PCR amplifications was 10 μl. 1 μl of DNA template was used per reaction. PCR amplification was done under different conditions for each primer set used. For the Montoncoup/Dwnmont2 primer set, initial denaturation was at 94 °C for 3 min, then 29 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 5 min. For the Montoncoup5/Montoncodwn1 primer set, initial denaturation was at 94 °C for 3 min, then 29 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 75 s, followed by a final extension at 72 °C for 5 min. A 5 μl aliquot of the amplification products were fractionated by electrophoresis on an 1.0% agarose gel in 1X TAE (48 mM Tris-acetate, 1 mM EDTA) buffer and visualized after staining with ethidium bromide (0.5μg/ml TAE) and UV transillumination. The molecular marker used was Promega 1 KB (Madison, WI). The gel image was taken with a Gel Logic100 system (Kodak). A total of five DNA samples could not be amplified and these males were removed from all analyses involving male *Xmrk* genotype (Table 1). The penetrance (complete/ incomplete) of the Sc phenotype was determined by comparing the Sc phenotype of each individual male against his *Xmrk* genotype from these PCR screening results.

Mirror image stimulation

Standard mirror image stimulation (MIS) tests were used to determine if the Sc phenotype (analysis 1) or *Xmrk* genotype (analysis 2) was correlated with male aggression and to investigate whether the Sc pattern is perceived as a signal in agonistic encounters (analysis 3). Male MIS tests were only conducted once for each treatment because the results of these behavioral assays have been shown to be highly repeatable in *X. cortezi* and numerous other *Xiphophorus* species (Franck *et al.*, 1985; Moretz & Morris, 2003). Males with naturally occurring spotted caudal (Sc) were tested in19L experimental aquaria with a line drawn at one end of the tank delineating the 10 cm interaction zone. Each experimental tank had a small plastic plant placed outside of the interaction zone for refugia. Individual males were placed in the 19 L experimental tanks and visually isolated from one another. During this transfer, all males were anesthetized with tricane methanesulphonate (MS-222) in order to accurately measure them (SL) and collect information about the individual's phenotype.

Spotted caudal (Sc) pigment pattern and vertical body bars were scored, the latter of which has previously been shown to influence male aggression in *X. cortezi* (Moretz, 2005). All males were in isolation for a minimum of 2 days (Mean = 8.26; Min = 2, Max = 34) before the start of the test, which allowed the males to establish residency in the experimental tank (Moretz & Morris, 2003). The testing procedure consisted of placing a mirror at one end of an individual's tank and recording the number of displays and bites directed at the mirror image over a five minute trial period. The mirror was placed thirty minutes after the painting manipulations (either mock water or dye). The five minute trial period began as soon as the individual entered the interaction zone and directly faced his image. Interaction time was recorded and defined as the time that an individual spent within the 10 cm interaction zone either displaying, biting, swimming back and forth in front of his image, or simply facing his mirror image. Two displays were recorded: lateral display, a lateral orientation of the body while quivering (Ryan & Causey, 1989); vertical headstand, head tilts downward until body is at ~45° angle with the substrate (Lyons & Morris, 2008). Both of these displays are common in *X. cortezi* male agonistic confrontations (Moretz & Morris, 2003). Finally, the number of bites directed at the mirror image was also recorded during the trial period.

It was necessary that the males collected in the field without naturally occurring Sc saw both a stimulus with the Sc pattern and a stimulus without the Sc pattern. Therefore, in one treatment, a temporary Sc pattern was applied to these males lacking the Sc phenotype using Dr. Naylor's Blue-Kote antiseptic dye (H. W. Naylor Co., Inc., Morris, NY; Hoefler & Morris, 1999) to approximate the average size of the Sc pattern for that male's population. In the case of Tecolutlo, in which all individuals collected lacked Sc (Table 1), an average sized Sc phenotype was painted based upon the other four populations. In trials where males were not to receive a dye painting of Sc, these males were painted with water to control for handling effects. Care was taken to ensure that handling time (~ 40 seconds) was consistent between treatments (painting vs. mock painting) and across individuals. Previous work has demonstrated that this technique neither harms the fishes nor alters their behavior (Hoefler & Morris, 1999) and that *X. cortezi* did not differ in their response to phenotypes painted with this dye and the natural phenotypes (Hoefler & Morris, 1999). The two tests conducted on males without the Sc phenotype, one test as 'painted Sc' phenotype and the other as 'no Sc' phenotype, were performed using the same methodology as performed on naturally occurring Sc males described above. Trials in which males without Sc were either painted or mock painted were conducted at least two weeks apart in order to reduce the influence of the first trial experience. Two weeks has been determined to be sufficient to reduce the effects of prior encounters (Moretz & Morris, 2003). Treatment order was randomized.

Statistical analyses

The possible influence of the male's resident population on the four recorded aggression variables was assessed using Kruskal-Wallis ANOVAs. However, because this question concerns differences between populations and not between treatments, there were three data sets for this initial analysis only (natural Sc, painted Sc, and male response to Sc). In other words, analyses were conducted within treatment (phenotype) groups and not across them. For all three of these data sets, the four aggression metrics (*i.e.*, interaction time, headstand displays, lateral displays, and bites) were analyzed in separate ANOVAs with population as a fixed factor. Because in the male response to Sc comparison (analysis 3) each male was tested twice and Kruskal-Wallis ANOVAs are univariate, I used the difference in the numbers of displays/bites performed in the natural state (No Sc) versus the painted Sc state as the dependent variable for the Kruskal-Wallis ANOVAs.

Analysis 1: Aggression and the Sc phenotype—In the assessment of male aggression using mirror image techniques (analyses 1 and 2), it is critical to ensure that

males are given the same stimulus. Therefore, in the two analyses of male aggression, males always saw an Sc patterned male when looking at the mirror (either naturally occurring Sc or artificially applied Sc) and their differences in aggression to that stimulus could be quantified. To determine if there was a correlation between the natural expression of the Sc phenotype and male aggression, the results of the mirror image trials for naturally occurring Sc males were compared against the results of painted Sc males (males that did not have natural Sc expression). Nonparametric Mann Whitney U tests were used to compare the mean aggression levels of naturally occurring Sc males to males without the Sc phenotype.

Analysis 2: Aggression and the Xmrk genotype—Because the Sc pattern has incomplete penetrance (Kallman, 1971), it is possible for the painted Sc males in analysis 1, which lacked natural Sc expression, to have the *Xmrk* genotype (see Table 1). Given a goal of this study was to determine if there was a correlation between the *Xmrk* oncogene and male aggression, these same data were also analyzed according to whether or not males had the *Xmrk* genotype (*i.e.*, irrespective of whether they had a natural Sc phenotype or a painted Sc phenotype). Nonparametric Mann Whitney U tests were used to compare the mean aggression levels of males with the *Xmrk* genotype to wildtype (*Xmrk* deficient) males.

Analysis 3: Male response to the Sc phenotype—To determine if the Sc phenotype is a visual signal used in male agonistic encounters one must account for individual variations in male aggression. Therefore, *X. cortezi* males without naturally occurring Sc were tested twice using mirror image stimulation, once with a temporarily applied Sc pattern and a second time in their natural non-Sc state after receiving a mock water painting. Thus, individual males served as their own controls. Each male's aggression towards their artificially painted Sc image and their natural non-Sc image were compared to determine if males alter their response when viewing an Sc patterned male. Wilcoxon signed ranks test were calculated to determine if there is a difference in the aggressive responses of males when viewing either their painted Sc image or their natural non-Sc image.

All statistical analyses were conducted using SPSS 16.0.1.

Results

Kruskal-Wallis ANOVAs found that there was no effect of population on any of the four dependent variables recorded for either the naturally occurring Sc males (headstands: X^2_2 = 2.832, P = 0.243; lateral displays: X^2_2 = 2.809, P = 0.245; bites: X^2_2 = 1.49, P = 0.475; interaction time: X^2_2 = 3.817, P = 0.148) or the painted Sc males (headstands: X^2_2= 4.764, P = 0.092; lateral displays: X^2_2 = 0.079, P = 0.961; bites: X^2_2= 1.177, P = 0.555; interaction time: X^2_2 = 0.562, P = 0.755). In the response to Sc comparison, males from different populations again did not differ in their aggressive responses for all four variables measured (headstands: X^2_4 = 3.799, P = 0.434; lateral displays: X^2_4 = 0.769, P = 0.943; bites: X^2_4= 7.961, P = 0.093; interaction time: X^2_4= 4.873, P = 0.301). Thus, the amount of aggression for each treatment was consistent across populations. Because male aggression did not vary across the five populations sampled, the data were pooled for each of the four variables in analyses 1, 2, and 3.

Penetrance of the Sc phenotype

X. cortezi males without phenotypic expression of Sc can have the associated *Xmrk* oncogene (*i.e.*, incomplete penetrance; Table 1). Incomplete penetrance of Sc was observed in four of the five populations and therefore appears to be widespread within the geographic distribution of *X. cortezi*.

Analysis 1: Aggression and the Sc phenotype

Naturally occurring Sc males spent significantly more time interacting with their mirror image than non-Sc males painted with the Sc pattern (Table 2; $Z = -2.341$, $P = 0.019$). Males with naturally occurring Sc also bite more at their image than painted Sc males (Fig. 2A; mean ± SE, natural Sc males = 7.3 ± 2.28, painted Sc males = 3.06 ± 1.03; $Z = -2.095$, $P = 0.036$). There was no difference in the number of displays performed by natural Sc males and painted Sc males (mean ± SE; headstand displays: natural Sc males = 1.2 ± 0.53, painted Sc males = 1.77 ± 0.49; $Z = -0.242$, $P = 0.808$; Fig. 2B, lateral displays: natural Sc males = 1.15 ± 0.37, painted Sc males = 0.71 ± 0.22; $Z = -1.523$, $P = 0.128$).

Analysis 2: Aggression and the *Xmrk* genotype

Xmrk males spent more time interacting with their image than wildtype (*i.e.*, *Xmrk* deficient) males (Table 2; $Z = -3.06$, $P = 0.002$). Males with the *Xmrk* genotype also bit more (Fig. 2A; mean ± SE, *Xmrk* males = 6.23 ± 1.59, wildtype males = 1.58 ± 0.74; $Z = -2.264$, $P = 0.024$) and performed more lateral displays (Fig. 2B ; mean ± SE, *Xmrk* males = 1.08 ± 0.26, wildtype males = 0.54 ± 0.31; $Z = -2.146$, $P = 0.032$) at their image than wildtype males. There was no difference in the number of headstands performed by *Xmrk* and wildtype males (mean ± SE, *Xmrk* males = 2.03 ± 0.52, wildtype males = 1.17 ± 0.61; $Z = -1.351$, $P = 0.177$). It is noteworthy that there was no significance difference in the aggression levels of *Xmrk* males with naturally occurring Sc and *Xmrk* males who did not naturally express Sc but were painted with Sc (Mann-Whitney U test: headstands: $Z = -0.903$, $P = 0.367$; lateral displays: $Z = -0.362$, $P = 0.717$; bites: $Z = -0.887$, $P = 0.398$; interaction time: $Z = -0.746$, $P = 0.455$). This suggests that the increased aggression of males with the *Xmrk* genotype is not due to the process responsible for the physical expression of Sc. The relationship between male aggression and *Xmrk* also cannot be explained by the presence or absence of vertical body bars in *X. cortezi* males, which has previously been shown to influence aggression (Moretz & Morris, 2003;Moretz, 2005). Males with vertical bars did interact more than barless males (Mann-Whitney U test: $Z = -2.290$, $P = 0.022$; data not shown) however there was no significant difference in the three aggression variables measured between the barred and barless morphs (Mann-Whitney U test: headstands: $Z = -1.364$, $P = 0.172$; lateral displays: $Z = -0.147$, $P = 0.883$; bites: $Z = -1.820$, $P = 0.069$). Furthermore, the *Xmrk* genotype and the vertical body phenotype were not correlated within individuals (Pearson correlation test: $r_{64} = -0.017$, $P = 0.894$).

Analysis 3: Male response to the Sc phenotype

In the examination of male response to Sc, *X. cortezi* males bit more at their natural non-Sc image than at their painted Sc image (Fig. 3A; mean ± SE, non-Sc image = 11.46 ± 1.85, Sc image = 5.46 ± 1.04; $Z = -3.368$, $P = 0.001$). Males also performed more lateral displays towards their natural non-Sc image than their painted Sc image (Fig. 3B; mean ± SE, non-Sc image = 3.55 ± 0.54, Sc image = 2.05 ± 0.42; $Z = -2.544$, $P = 0.011$). Male response to Sc was not different for the number of headstand displays performed (mean ± SE, Sc image = 3.18 ± 0.54, non-Sc image = 2.48 ± 0.47; $Z = -1.080$, $P = 0.28$) or interaction time (Table 2; $Z = -1.767$, $P = 0.077$).

Discussion

This study found that *X. cortezi* males with naturally occurring Sc bit more and performed more agonistic displays at their image than non-Sc males painted with the Sc pattern. Furthermore, *Xmrk* males, regardless of whether they naturally expressed the Sc pattern, were also more aggressive than *Xmrk* deficient males. This result suggests that genetic factors linked to the *Xmrk* oncogene on the sex chromosomes are underlying the observed male aggression (see below) and not the genomic region surrounding the autosomally

encoded Sc phenotype. Differences in male aggressive response towards the Sc pattern indicate that the Sc phenotype does function as a signal in *X. cortezi* male agonistic encounters. *X. cortezi* males without naturally occurring Sc, regardless of their *Xmrk* genotype, performed significantly fewer lateral displays and bites when viewing their painted Sc mirror image as compared to their natural non-Sc image. This result implies that the Sc signal is sufficient to convey information about individual male aggression and that it likely plays a role in determining the outcome and duration of male encounters. Collectively, the findings of this study demonstrate that individual males with the *Xmrk* genotype experience a competitive advantage in male-male competition at the potential expense of a reduced reproductive lifespan due to melanoma formation. The evolutionary persistence of *Xmrk* oncogene within *X. cortezi* likely results from the benefits of increased male attractiveness (Fernandez & Morris, 2008) and aggression associated with genetic factors linked to *Xmrk* genotype outweighing the potential costs of decreased offspring viability due to genetic incompatibility (Schartl *et al.*, 1998) and a decreased reproductive lifespan.

The *Xmrk* oncogene encodes for an epidermal growth factor receptor protein (*Egfr* paralog) that is unlikely to be responsible for the observed increased aggression in *X. cortezi* males with this genotype. However, the *Xmrk* oncogene is in close genomic proximity to a number of genes important in sexual selection (Froschauer *et al.*, 2002), including but not limited to the master sex determining gene (*SD*), the pituitary locus that determines size at sexual maturation (P alleles; Kallman *et al.*, 1973), and the red-yellow locus (*RY*; Kallman, 1975). For example, *RY* encodes for red, yellow, brown, and orange coloration on the body and fins of *Xiphophorus*, and female preferences have been documented for such color morphs (Basolo & Trainor, 2002; Franck *et al.*, 2003). In addition, a recent study found this region also encodes a functional copy of *Mc1r* (melanocortin type 1 receptor; Selz *et al.*, 2007), which promotes melanogenesis in skin melanocytes (Metz *et al.*, 2006) and has elevated expression levels in *Xiphophorus* melanomas (Selz *et al.*, 2007). The close proximity of these loci to the *Xmrk* oncogene combined with the decreased rate of recombination between sex chromosomes (Bergero & Charlesworth, 2008) provides an ideal environment for genetic hitch-hiking (Maynard Smith & Haigh, 1974). Although we known relatively little about melanocortins and their receptors in teleosts fish compared to mammals (Gantz & Fong, 2002; Metz *et al.*, 2006), melanocortins do promote androgen production in the adrenal cortex in mammals (Gantz & Fong 2002) and it is believed that up to seven copies of *Mcrs* are found within the immediate vicinity of *Xmrk* (Froschauer *et al.*, 2002). Therefore, melanocortin receptors in this region could underlie the increased aggression of males with the *Xmrk* genotype if linkage disequilibrium occurs between these loci. However, detailed genetic mapping of this region and population genetic studies are necessary in order to elucidate the precise genetic mechanism responsible for the increased aggressiveness of *Xmrk* males in this study.

The occurrence of melanomas early in the lifespan of *X. cortezi* males (~7–12 months; Kallman, 1971; Schartl *et al.*, 1995) and later in the lifespan of females is consistent with the seminal theoretical work proposed by Williams (1957) on antagonistic pleiotropy and senescence. Williams (1957) argues that a gene conferring an advantage at one age and a disadvantage at another will depend not only on the magnitude of these effects but also the timing of these effects. Within *Xiphophorus*, females (unlike males) have indeterminate growth (Reznick & Miles, 1989) and therefore female fecundity and total reproductive potential increases with age (Williams, 1957). However, Williams argues that the potential male reproductive fitness declines with age once sexual maturity is reached in organisms in which males have determinate growth (Williams, 1957). Because of these life history characteristics, selection should act in different ways to maximize the total reproductive probability of each sex (Williams, 1957). The occurrence of melanomas early in females would be costly (more so than in males) and should be selected against. However, selection

favors a strategy in males that maximizes mate acquisitions and direct benefits as soon as sexual maturity is reached because thereafter the risk of mortality increases and reproductive fitness declines (Williams, 1957). The mating advantages associated with Sc and *Xmrk* in *X. cortezi* males, via mate preferences (Fernandez & Morris, 2008) and male-male competition (this study), can therefore offset the occurrence of malignancies earlier in males as long as sexual maturity is reached. The prevalence of non-hybrid melanomas across species also conforms to the theoretical work proposed by Williams (1957). Under laboratory conditions, melanomas occur in approximately 42% of *X. variatus* but only occur after 18 months of age (Kazianis & Borowsky, 1995) when selection against senescence is relaxed. However, the frequency of melanomas in *X. cortezi* in the laboratory and in nature is less than 10 % (laboratory: Kallman, 1971; Schartl *et al.*, 1995; nature: Fernandez & Morris, 2008) due, at least in part, to their occurrence earlier in adulthood.

The status-signaling hypothesis proposes that variation in trait morphology (*e.g.*, coloration) reflects an individual's ability to win agonistic contests (Rohwer, 1975, 1977). The use of 'badges of status' to reliably signal dominance in social arenas is well documented within the literature for a wide variety of taxa (primates: Gerald, 2001; Setchell & Wickings, 2005; birds: Rohwer, 1977; Møller, 1987; lizards: Thompson & Moore, 1991). Frequently, the expression of such traits directly covaries with testosterone levels (Rand, 1992; Sinervo *et al.*, 2000; Setchell & Dixson, 2001; Cox *et al.*, 2005), and it is not surprising that dominance status is often positively correlated with levels of aggression. The costs of producing and carrying such signals (Zahavi, 1975), as well as the potential direct costs associated with being dominant (Hannes *et al.*, 1984; Creel, 2001; Castro *et al.*, 2006), can maintain the honesty of these signals. These costs are then offset by an individual's greater access to contested resources (food: Maclean & Metcalfe, 2001; mates: Morris *et al.*, 1992; Rantala & Kortet, 2004). Because Sc is associated with increased aggression in *X. cortezi* males, and males differentially respond to this signal, the Sc phenotype might serve as a badge of status according to the status-signaling hypothesis. However, one important criterion for the status signaling hypothesis is that variation in a signal can accurately convey information about dominance, thereby allowing subordinate individuals to use this signal to avoid potentially costly agonistic encounters (Rohwer, 1982; Saner & Camerino, 1998). Although it is clear that Sc varies dramatically in size not only within but also between populations (Fig. 1; Kallman, 1971), the relationship between the extent of Sc expression and dominance and/or aggression has not been formally established. The size of Sc (and other M patterns within *Xiphophorus*) is determined by the expression levels of *Xmrk* (Weis & Schartl, 1998; Meierjohann & Schartl, 2006) and M patterns do increase in size after exposing *Xmrk* individuals to elevated levels of testosterone (Schartl *et al.*, 1981; Schartl *et al.*, 1982; Schartl *et al.*, 1995). Lastly, although aggression is some taxa is not correlated with testosterone levels (Steklis *et al.*, 1985; Silverin *et al.*, 2004), Hannes (1986) demonstrated in the swordtail *X. helleri* that the number of bites and sigmoid threat displays in male agonistic encounters were positively correlated with the testosterone concentrations in blood and whole body tissues. Therefore, it is not unreasonable to suggest a positive relationship exists between the increased aggression levels of Sc and/or *Xmrk* males detected in this study and the degree of Sc expression; however, future hormonal research needs to verify this in order to confirm the Sc M pattern is a badge of status in *X. cortezi*.

The use of male mirror image stimulation (as opposed to dyadic contests) to assess dominance has received some criticism because the outcomes of these different behavioral assays are not always consistent (Meliska *et al.*, 1980; Ruzzante, 1992; although see Holtby, 1992). For example, individual *Betta splendens* that differentially responded during mirror image tests became indistinguishable in their aggression measures when using dyadic contests (Meliska *et al.*, 1980). However, recent behavioral research conducted on *X. cortezi*, which focused on the vertical bar phenotype, found that *X. cortezi* males are

consistent in their aggression measures across dyadic contests and male mirror image stimulation (Moretz & Morris, 2003; Moretz, 2005). Most importantly for the implications of this study was the finding that the number of bites (an aggression metric used in the current study) was the best predictor of winners in *X. cortezi* dyadic contests (Moretz, 2005). Furthermore, male MIS is the only method suitable to assess individual aggression levels (Franck *et al.*, 1985; Rowland, 1999). This is because MIS, unlike simultaneous male contests or dyads, provides instantaneous feedback that is not dependent on intermale behavioral interactions while controlling for potential confounds such as body size and coloration (Rowland, 1999). This methodology also permits experimental manipulations and sequential testing in order to assess behavioral responses to a signal of interest while concurrently controlling for individual variation (*e.g.*, male response to Sc in this study).

Xiphophorus has proven to be an ideal system in which to study sexual selection theory. In addition, this genus has served as the premiere animal model for studying the genetic basis to skin cancer for more than 80 years. The findings of this study indicate that merging these fields of scientific investigation can be very insightful and establishes *Xiphophorus* as an excellent system to study the evolutionary biology of cancer and life history trade-offs as they relate to the *Xmrk* genotype. Furthermore, bridging this gap is essential in order to further our understanding of the evolutionary relationship between the *Xmrk* genotype and the associated phenotype in this animal model.

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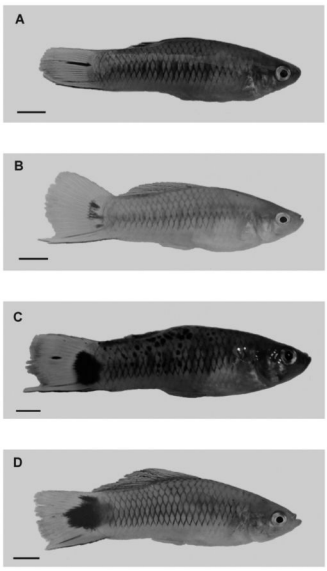

Figure 1.
These *X. cortezi* males (Panels A–D) were all collected on the same day from the Conchita collection site (San Luís Potosí, Mexico). This amount of variation in pattern size and saturation is typical across *X. cortezi* populations. Scale bar: 5 mm.

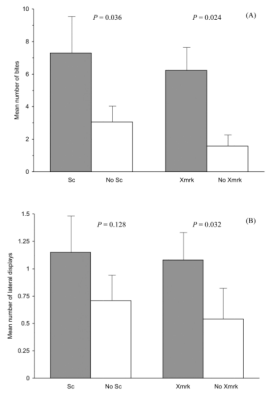

Figure 2.

The number of bites (A) and lateral displays (B) performed by *X. cortezi* males during the five minute MIS trials. The results of analysis 1 (aggression and Sc phenotype) are presented on the left side of each bar graph (Sc/no Sc) and the results of analysis 2 (aggression and the *Xmrk* genotype) are presented on the right side of each graphic (*Xmrk*/no *Xmrk*). As a reminder in both analyses 1 and 2, males were responding to the same stimulus, their mirror image with the Sc phenotype (*i.e.*, no Sc males had the Sc phenotype painted on). Bars represent the mean ± SE.

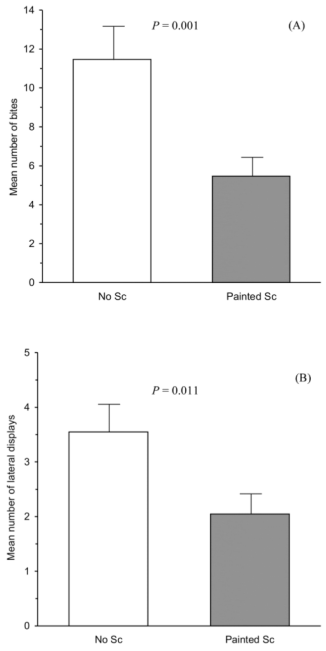

Figure 3.
The number of bites (A) and lateral displays (B) performed by *X. cortezi* males towards either their non-Sc image (white bars) or their painted Sc image (grey bars) during the five minute MIS trials. Bars represent the mean ± SE.

Table 1

The frequency of the spotted caudal (Sc) phenotype and the *Xmrk* genotype in *X. cortezi* males from the five populations in this study. Note the increase in the frequency of *Xmrk* males compared to Sc males in populations due to the incomplete penetrance of the Sc pattern.

Population	Total	Sc males	Non-Sc males	*Xmrk*	No *Xmrk*
Chalpuhuacanita	23	7	16	9	14
Conchita	30	10	20	23	7
Tanute	15	3	12	8[a]	3[a]
Tecolutlo	13	0	13	0[b]	12[b]
San Martín	26	0	26	1	25

[a] Non-viable DNA for four males

[b] Non-viable DNA for one male

Table 2

The amount of time males spent interacting with their mirror images in the MIS trials. Data are mean values ± SE.

Analysis	Male condition	Interaction time (sec)	Probability
1	Natural Sc Painted Sc	219.7±23.1 141.0±18.0	$P = 0.019$
2	*Xmrk* No *Xmrk*	198.5±19.1 110.8±21.6	$P = 0.002$
3	No Sc Painted Sc	188.8±12.4 162.5±12.7	$P = 0.077$