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This dissertation entitled

SELECTION FOR THE XMRK ONCOGENE IN XIPHOPHORUS CORTEZI

by

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has been approved for

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ABSTRACT

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SELECTION FOR THE XMRK ONCOGENE IN XIPHOPHORUS CORTEZI (142 pp.)

Director of Dissertation: Molly R. Morris

This dissertation examines sexual selection as a mechanism underlying the continued evolutionary maintenance of the Xmrk (Xiphophorus melanoma receptor kinase) cancer gene within the Xiphophorus melanoma model. Additionally, I expand this animal model to include Xiphophorus nezahualcoyotl (Order: Cyprinodontiformes, Family: Poeciliidae) as a species capable of non-hybrid melanoma formation. I use the well-studied Northern swordtail, Xiphophorus cortezi, collected from six localities throughout its geographic distribution to address whether the pigment pattern from which melanomas form (spotted caudal, Sc) and/or the Xmrk oncogene responsible for melanomas within Xiphophorus are advantageous in the acquisition of mates.

Specifically, I address the following questions: 1) Is there a relationship between male aggression levels and the Sc phenotype and/or Xmrk genotype within individual males; 2) Does male aggressive response differ based upon the presence of the Sc phenotype; 3) Do females preferentially associate with Sc patterned males over non-Sc males or with larger Sc patterned males to size-matched males with smaller Sc patterns; and 4) Does the frequency of the Sc phenotype or the Xmrk genotype across the six populations influence male aggression levels or female mate choice decisions?
The results of mirror image trials found that the Sc macromelanophore pattern as well as the \textit{Xmrk} oncogene (regardless of the presence of Sc) is correlated with increased aggression. In addition, Sc appears to function as a visual signal in male agonistic encounters because male aggressive response decreases when viewing their Sc image as compared with their non-Sc image. The frequency of \textit{Xmrk} in males across populations ranged 0\% to 87\%. However, there was no difference in the aggression levels of males with Sc and/or \textit{Xmrk} from each population thus the frequency of \textit{Xmrk} within a population does not directly influence individual levels of male aggression. \textit{X. cortezi} females from three populations, located in separate drainages that are genetically divergent, prefer to associate with Sc patterned males to non-Sc males. Moreover, \textit{X. cortezi} females prefer males with an enhanced Sc pattern, which would occur during melanoma formation, to males with a reduced Sc pattern. However, unlike male aggression, there was variation in female preference for Sc males and it appeared to be influenced by the frequency of \textit{Xmrk} in the population. Females from one population, which had the highest frequencies of Sc and \textit{Xmrk} in females, discriminated against Sc patterned males and preferred to associate with non-Sc males. These results suggest there is a negative relationship between the strength of female preference for Sc and the frequency of \textit{Xmrk} in females across populations. Because offspring with two copies of \textit{Xmrk} have reduced fitness, and these offspring are more likely to occur in populations in which the frequency of \textit{Xmrk} in females is high, females can increase their reproductive fitness by avoiding males with Sc (and therefore \textit{Xmrk}) in these populations.
The findings of this dissertation have several important implications for the *Xiphophorus* melanoma model. First, non-hybrid melanomas occur in more *Xiphophorus* species than initially realized and may be more biologically relevant within *Xiphophorus* than melanomas formed via interspecific hybridization. Second, the *Xmrk* oncogene is associated with increased male aggression and thereby provides a competitive advantage for individuals in male-male competition. In addition, the macromelanophore patterns associated with the *Xmrk* oncogene can serve as signals in these male agonistic encounters. Third, female mate choice for the *Xmrk* associated melanin patterns plays an important role in the evolutionary maintenance of this oncogene. Finally, the relative frequency of *Xmrk* within each sex of a population does influence female mating decisions and is likely responsible for the continued polymorphism of *Xmrk* in all *Xiphophorus* that have retained this cancer gene. Collectively, the research presented in this dissertation demonstrates that sexual selection is important in explaining the persistence of *Xmrk* within this system.

Approved: ________________________________

Molly R. Morris

Associate Professor of Biological Sciences
To everyone and anyone who, over the last thirty-one years, made this document reality.
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Without question the first person I wish to acknowledge is my advisor and mentor, Dr. Molly R. Morris. The reason is simple: none of this would have happened without her giving me the opportunity to do so. Knowing what I know now about the process of graduate school (and the investment an advisor makes), I am truly amazed that she chose to take me on. I was five years removed from undergraduate education (with mediocre grades) and the only research experience I had was collecting data on primate foraging behavior in the field (I had never heard of a swordtail!). Yet, she read between the lines and believed in my potential to excel, which in turn has always led me to believe in myself. Thank you Molly. Thank you for allowing me the freedom to grow and develop as a scientist, yet always being there when I fell or needed guidance. I truly believe the latitude you give us, your students, is paramount to the success we attain.

The integrative nature of this dissertation (sexual selection, evolutionary biology, and molecular biology of cancer) frequently pushed me beyond my training as a behaviorist and forced me to glean information and expertise from others with the Department of Biological Sciences at Ohio University. At the top of this list is Dr. Soichi Tanda. I am indebted to Dr. Tanda for his assistance and devotion in cracking the molecular code of this cancer gene (collectively, we ordered 53 custom primers before finally obtaining a primer set capable of screening for this cancer gene). Thank you for opening up your laboratory and imparting me with such great molecular techniques. Your optimism and humor in this endeavor proved vital in this last year. I would also like to
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Throughout my tenure at Ohio University, my family has sacrificed spending time with me as my studies progressed. For me, as this immersion took place, the separation became easier and easier. This was not the case for them. Because the enticements of scientific investigations are unfamiliar to them, my persistent absences came with more and more bewilderment. I would like to thank all of them for saying they understand when I know they really didn’t. In my mind, such support is the definition of family.

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## INTRODUCTION

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GENERAL INTRODUCTION

Historically, the discussion of an individual’s genetic predisposition to cancer involved their exposure to environmental risk factors (e.g., mutagens), which resulted in a somatic mutation event occurring during their lifetime. Thus, the risk of developing cancer could involve DNA that was altered because of mutagenic exposure although such mutations responsible for the cancer under these circumstances are not passed onto subsequent generations. However, genes directly associated with cancer formation (oncogenes) have recently been discovered that are passed onto subsequent generations (for review Ross et al. 1998; Crespi and Summers 2006). Such oncogenes represent an evolutionary paradox. If the oncogenes are deleterious and therefore reduce viability and/or reproductive fitness, these cancer-causing genes must provide some benefit to the organisms if they are to be conserved over time (especially if individuals are in a resource-limited environment and there is competition). Alternatively, the oncogenes could induce cancer formation post-reproduction during senescence and have little, if any affect, on the reproductive fitness of that individual.

The discovery of oncogenes has led to the emergence of a new field of scientific investigation: the evolutionary biology of cancer (for review see Graham 1992; Greaves 2000; Crespi and Summers 2006). However, the mechanisms by which cancer evolves and persists in natural systems have been difficult to ascertain. One example, and the focus of this dissertation, is the Xmrk oncogene (*Xiphophorus* melanoma receptor kinase) within the genus *Xiphophorus* (commonly known as the swordtails and platyfishes). The
Xmrk oncogene arose through a gene duplication event and has been maintained for several million years despite being deleterious (Schartl et al. 1995; Weis and Schartl 1998) and located in an extremely unstable genomic region (Volff et al. 2003; for review see Meierjohann and Schartl 2006). Phylogenetic analysis and similarities in the molecular structure of Xmrk across species indicate that the evolution of Xmrk predates the divergence of the genus (i.e., ancestral), with subsequent losses in numerous species (Weis and Schartl 1998; Meierjohann et al. 2004).

A simple two-locus model, which includes Xmrk and its autosomal suppressor gene (unknown R), has been proposed as the genetic mechanism underlying melanoma formation in Xiphophorus (Meierjohann et al. 2004). The expression of Xmrk oncogene results in the formation of melanomas that originate from specific patterns composed of a certain type of pigment cell, macromelanophores. At least 10 of 26 described Xiphophorus species have black pigment patterns comprised of macromelanophores (Weis and Schartl 1998; Franck et al. 2001). In eight of these 10 Xiphophorus species with macromelanophores, hybridization can lead to either enhancement of the patterns (melanosis) or the development of severe malignant melanomas (Weis and Schartl 1998). For decades it was believed that hybridization, in which the sex-linked Xmrk and the autosomal suppressor R become disassociated via chromosome segregation, was the only way to get melanoma formation in Xiphophorus. However, Kallman (1971) and Borowsky (1973) presented evidence of spontaneous nonhybrid melanoma formation in two species of wild caught Xiphophorus housed in their laboratory (X. cortezi: Kallman 1971; X. variatus: Borowsky 1973). Under both circumstances, hybrid and nonhybrid, the
molecular pathway leading to melanoma formation is the same: the overexpression of \textit{Xmrk} within the Ras/Raf/MAPK signaling pathway results in dedifferentiation and uncontrolled cellular proliferation with subsequent neoplastic progression initiating from the associated macromelanophore pattern (Schartl et al. 1995; Meierjohann and Schartl 2006).

Of the two species documented to form nonhybrid melanomas at the conception of this dissertation, \textit{X. cortezi} poses the most intriguing questions for an evolutionary biologist like myself. Unlike \textit{X. variatus}, which only develop melanomas later in life (i.e., >18 months old age during senescence; Schartl et al. 1995), \textit{X. cortezi} develops malignant melanomas as early as 7 months (Kallman 1971). In addition, the incidence of melanoma formation in \textit{X. cortezi} is higher in males than females (melanomas inflict the sexes relatively equally in \textit{X. variatus}; Schartl et al. 1995). Finally, the formation of melanomas has been suggested to occur most frequently in ‘sexually active males of high social rank’ (per Schartl et al. 1995). All of this indicates that melanomas in \textit{X. cortezi} may be more biologically relevant than in \textit{X. variatus}, and implies that sexual selection might play a role in the maintenance of this oncogene.

Because it is central to understanding the findings of this dissertation I want to explain what is currently known about the \textit{Xmrk} oncogene and the Sc macromelanophore pattern that serves as the site of melanoma formation in \textit{Xiphophorus cortezi}. Males and females of this species are polymorphic for both Sc and \textit{Xmrk}, meaning that some individuals have these traits and others do not. Melanomas in this species only originate from Sc macromelanophore pattern in the caudal fin and therefore Sc is the only
macromelanophore pattern associated with the Xmrk oncogene in this species (the two other macromelanophore patterns have never been documented to form melanomas; Kallman 1971). Xmrk is an essential component for the expression of the Sc phenotype in X. cortezi (Schartl et al. 1995; Chapter 4) and can be located on the X and/or Y chromosomes (Froschauer et al. 2002). Therefore, all X. cortezi with the Sc phenotype have the Xmrk genotype and melanomas can form in both sexes. However, the Sc pattern has incomplete penetrance, which means that individuals can have the gene for Sc but not express it as a phenotype (i.e., Sc pattern). Thus, individuals without the Sc pigment pattern can have the Xmrk oncogene. The gene for Sc, unlike Xmrk, has not yet been described at the genetic level.

There are two components of sexual selection: female male choice and male-male competition (Andersson 1994). Previously, X. cortezi has been shown to use the melanin pattern vertical body bars in both mate choice and male-male competition (Morris 1998; Morris et al. 2003; Moretz and Morris 2003). The primary goal of this dissertation was to investigate the potential mating advantages of the spotted caudal (Sc) macromelanophore and the associated Xmrk oncogene in X. cortezi. Because X. cortezi females prefer more melanin pigmentation in males (Morris 1998), female mate choice could play an important role in the maintenance of Xmrk through the phenotypic expression of the Sc pattern. The formation of melanomas due to Xmrk would increase the size of Sc in males and therefore overall body pigmentation. In males, the Sc pattern could serve as a signal in agonistic encounters similar to the vertical body bar pattern (Moretz and Morris 2003). In addition, the Xmrk oncogene, regardless of the expression of Sc, could influence male
aggression. If Xmrk is correlated with increased aggression, Xmrk males could gain mating advantages by chasing subordinate (i.e., Xmrk deficient) males away from females and as a result have greater access to females. To summarize, the Sc phenotype and the associated Xmrk oncogene in X. cortezi may be maintained under sexual selection through mate choice and male-male competition.

Chapter 1 (Fernandez and Bowser 2008) provides gross and histological evidence of non-hybrid melanoma formation in two wild-caught Northern mountain swordtails, Xiphophorus nezahualcoyotl that had not been previously documented. Each fish represented a novel and distinct case of melanoma formation. During initiation, one case showed increases in melanin expression from a micromelanophore pattern associated with the sword (sword stripes), and the other case showed melanin enhancement associated with the spotted-side macromelanophore pattern on the flanks of the fish. Both cases have spindle cell type melanomas located in the epidermis, dermis, adipose tissue, and underlying muscle in several locations on each fish. The melanomas in both fish were attributed to the Xmrk oncogene based upon gross and histological similarities of the lesions (subsequent molecular screening confirmed this, Fernandez unpublished data). Therefore, this study expands the Xiphophorus melanoma model to include the origination of melanomas from micromelanophore patterns and Xiphophorus nezahualcoyotl as a species susceptible to nonhybrid melanoma formation.

Chapter 2 uses mirror image stimulation (MIS) to examine correlations between the Sc phenotype and the Xmrk genotype with male aggressive behaviors in Xiphophorus cortezi from five different populations. The results of MIS tests were analyzed in two
different comparisons: 1) across males to determine if the expression of spotted caudal (Sc) or the presence of \textit{Xmrk} affects male aggression and 2) within males to investigate the potential function of the Sc pattern in aggressive encounters. In the first comparison, the aggressive interactions of naturally occurring Sc males were paired against those of painted Sc males (males did not have natural Sc expression). Therefore, both males in this comparison were seeing the same image (Sc patterned male), and differences in their inherent aggression levels could be quantified. In the second comparison, male response to the Sc pattern was assessed within males that naturally lacked the Sc phenotype by comparing their aggressive responses to both their artificially painted Sc and their natural non-Sc image. I found that natural Sc males and males with \textit{Xmrk} (regardless of whether or not they expressed Sc) were more aggressive in these trials than those that lacked the Sc phenotype and the \textit{Xmrk} genotype. Males also responded less aggressively when viewing their image with Sc than without Sc indicating that Sc serves as a signal in agonistic encounters. Collectively, these findings indicate that \textit{X. cortezi} males with Sc and/or \textit{Xmrk} gain advantages in male-male competition and improve male reproductive fitness. Therefore, intrasexual selection is an important component in the continued evolutionary maintenance of the \textit{Xmrk} oncogene within \textit{Xiphophorus cortezi}.

Chapter 3 (Fernandez and Morris \textit{in press}) examines female’s preferences for the Sc phenotype in \textit{X. cortezi} males from three populations using standard dichotomous choice tests. Preference tests were also conducted on females from one population (Cebolla) to determine if they exhibited preferences for the size of the Sc phenotype in males. The study found that \textit{Xiphophorus cortezi} females from two of the three
populations prefer males with the Sc melanin pattern to males without this pattern. Females from the third population, which had the highest frequency of Sc in females, discriminated against Sc males, preferring non-Sc males. This suggests that the frequency of Sc (and therefore \( Xmrk \)) within a population is an important determinant to whether positive selection exists for the Sc phenotype and \( Xmrk \) genotype within \( X. cortezi \).

Choice tests for the size of Sc, found that females prefer males with an enhanced Sc to males with a reduced Sc pattern. Gene expression analysis confirmed the tissue specific expression of the \( Xmrk \) oncogene within the Sc pattern in \( X. cortezi \). This analysis determined that not only is \( Xmrk \) a functional oncogene (i.e., capable of transcription) but also that the expression of this gene is specific to the Sc pigment pattern. Because of the association of \( Xmrk \) with the Sc pigment pattern, and the fact that melanoma formation augments this visual signal, sexual selection influences the maintenance of this oncogene due to a mating preference for Sc as well as the exaggeration of this male trait. Decreases in the viability and fecundity of individuals due to \( Xmrk \) (Borowsky 1973; Schartl et al. 1995; Schartl et al. 1998) and subsequent melanoma formation appear to be mitigated via increases in mate acquisition.

The final chapter presents female preferences for the Sc pattern in three additional populations in \( X. cortezi \), which are located within a separate river drainage from those examined in Chapter 3. In addition, Chapter 4 estimates the frequency of Sc and \( Xmrk \) across all six natural populations of \( X. cortezi \) investigated in this dissertation. Whereas many studies have been devoted to the \( Xmrk \) oncogene within \( Xiphophorus \), no study has reported the frequency of this cancer gene within natural populations. The
results showed there was no preference for Sc in two of the populations examined. However, in the third population preferences for Sc were detected despite the absence of the Sc phenotype or $Xmrk$ genotype within this population. The ratio of males to females with $Xmrk$ across populations was either male biased or approximately equal (females biased not observed), ranging from 0% in one population to almost 70% in two of the populations. The frequencies of Sc and $Xmrk$ across the six populations, coupled with the female preference for these populations, suggest that $Xmrk$ can not be maintained at high frequencies in both sexes within a population despite the advantages it confers to males (male competition and mate advantages) and females (indirect fitness benefits; e.g., producing attractive sons). If $Xmrk$ is too common in both sexes, there is an increased likelihood of producing offspring with two copies of $Xmrk$ within that population and such offspring would have reduced viability and fecundity (Borowsky 1973; Schartl et al. 1995; Schartl et al. 1998).
LITERATURE CITED


Fernandez A. A. and M. R. Morris. in press. Mate choice for more melanin as a mechanism to maintain a functional oncogene. The Proceedings of the National Academy of Sciences of the United States of America.


CHAPTER 1

TWO CASES OF NON-HYBRID MELANOMA FORMATION IN

XIPHOPHORUS NEZAHUALCOYOTL
ABSTRACT

Gross and histological evidence is presented to document two novel and distinct cases of non-hybrid melanomas in wild-caught Northern mountain swordtails, *Xiphophorus nezahualcoyotl*. During initiation one showed increases in melanin expression from a micromelanophore pattern associated with the sword (sword stripes), and the other showed melanin enhancement associated with the spotted-side macromelanophore pattern on the flanks of the fish. Both cases have spindle cell type melanomas located in the epidermis, dermis, adipose tissue, and underlying muscle in several locations on each fish.
INTRODUCTION

*Xiphophorus* (Poeciliidae, Cyprinodontiformes) is a morphologically diverse genus of fish that has served as a model system for studies of sexual selection, sensory bias, and melanoma research among others. Over the last decade, the *Xiphophorus* melanoma model has been receiving increased attention due to the more than doubling of human malignant melanoma cases from 1973 to 1994 (Dennis 1999). As early as the 1920s, it was known that interspecific crosses between platyfish and southern swordtails could induce hereditary melanoma formation (classic Gordon-Kosswig cross; Gordon 1927; Kosswig 1928). It was later realized that non-hybrid melanoma formation was also possible in this system, having been documented in two species, Cortes swordtail, *Xiphophorus cortezi*, Rosen (Kallman 1971; Schartl et al. 1995) and Variable platyfish, *Xiphophorus variatus*, Meek (Borowsky 1973; Kazianis and Borowsky 1995; Schartl et al. 1995). Regardless of origin, by hybridization or non-hybridization, melanoma formation in this model has been attributed to derepression of the *Xiphophorus* melanin receptor kinase (*Xmrk*) and over expression of melanin in an associated pigment pattern that is closely linked to *Xmrk* (Schartl et al. 1995; Meierjohann et al. 2004). The macromelanophore determining locus, *Mdl*, is a separate genetic entity from *Xmrk* and encodes the pigment pattern that associates with *Xmrk* (Weis and Schartl 1998). The melanotic pattern that is the site of neoplastic progression varies among species but has always been a macromelanophore based pattern (hereafter M pattern), as opposed to a micromelanophore pattern (although see Schartl et al. 1995).
The seminal work of Weis and Schartl (1998) has been critical in categorizing which species have \textit{Xmrk} and M patterns, and for increasing our understanding of the relationship and interplay between the two loci. Their research found that 10 of 26 \textit{Xiphophorus} species possess different polymorphic M patterns encoded at the \textit{Mdl} locus. Crossing experiments between these 10 species with M patterns and species that lack M patterning revealed eight of the 10 developed malignant melanomas or at least enhanced expression of the M pattern (i.e., melanosis). Two species, Northern mountain swordtail (\textit{Xiphophorus nezahualcoyotl}, Rauchenberger, Kallmann and Morizot) and Green swordtail (\textit{Xiphophorus helleri}, Heckel), possessed an M pattern that showed no change (\textit{X. helleri}) or even reduced expression of the M pattern after hybridization (\textit{X. nezahualcoyotl}). \textit{Xmrk} genotyping of these 10 species found that the eight crosses with enhanced expression after hybridization had the \textit{Xmrk} allele, whereas \textit{X. nezahualcoyotl} and \textit{X. helleri} lacked the \textit{Xmrk} allele. From these findings, Weis and Schartl (1998) deduced that \textit{Xmrk} is intimately linked to the \textit{Mdl} locus because every species that had a copy of the \textit{Xmrk} allele also had a M pattern encoded at the \textit{Mdl} locus. However, the reverse was not true; not all species that had a M patterning necessarily had the \textit{Xmrk} oncogene (e.g., \textit{X. helleri}).

\textit{X. nezahualcoyotl}, a member of the Northern swordtail clade, has an M pattern (spotted-side, Ss). The Ss pattern in this species appears as numerous medium and large size black spots along the flanks of the body (Figure 1.1A) and has sex linked inheritance (Kallman 1983). \textit{X. nezahualcotoyl}, along with all sword-possessing \textit{Xiphophorus} species, have black pigmented marginal lines that run along the edges of the sword
(termed “sword stripes”) which are composed of micromelanophore pigment cells (Rosen 1960; personal observation). Here two cases were examined of increased melanin expression in wild caught *X. nezahualcoyotl* that had different points of origin: micromelanophore pattern sword stripes (case A) and macromelanophore pattern spotted-side (case B). Melanoma formation has not been documented in *X. nezahualcoyotl*. The goals of this study were to determine: 1) if observed superficial pigment enhancement was invasive; 2) the extent of this invasiveness if found that is melanosis or melanomas; and 3) the rate of progression in one case (A) in which sequential photographs had been taken.

**METHODS**

*Specimen collection and housing*

Both fish presented here were caught during the April 2004 field season from the El Salto River (San Luís Potosí, Mexico). Upon return to the US, the fish were kept separately in 19 l tanks at Ohio University and screened from other fish in the laboratory. Both fish were maintained under standard laboratory conditions: 12L:12D cycle, daily feeding with Tetramin® flakes, and a constant temperature of 22° C. Fish used as histological references for normal specimens were also collected from the El Salto River during the same field season (April 2004) and kept under the same conditions.
Digital photographs

Photographs of the two cases were taken with a Fuji Finepix S7000 camera under standard fluorescent lighting conditions. Pictures of case A were taken from 4 August 2005 through 12 October 2005 at approximately 17 day intervals (five days in total). Sequential photographs were taken to document melanoma progression based on superficial melanin pigmentation. The single photograph presented here of case B was taken on the 18 September 2006, the day this specimen was prepared for histology.

The digital photographs for case A were analyzed using imaging software in order to calculate the area of melanoma patterning on each day. Area calculator (Rasband 2000), a java-based plug-in implemented in Image J (version 1.37), was used to quantify the total melanin area associated with the pattern of interest after converting each image to an 8-bit file (i.e., greyscale). The “set scale” command was employed within Image J to take into account the scale of each image when determining the area of the pattern (mm²).

Tissue preparation and histology

For histological preparation, the two specimens of X. nezahualcoyotl were killed by methane tricaine sulfonate (MS222) overdose, fixed in 10 % neutral buffered formalin and decalcified with sodium EDTA. They were then trimmed in the transverse plane to produce five cross sections representative of the head as well as four sections of the body. The trimmed transverse body sections were then embedded in paraffin, sectioned and stained with haematoxylin and eosin (Luna 1968). The four, apparently normal, reference
X. nezahualcoyotl were processed in a similar manner. All observed microscopic lesions were documented and melanomas were classified according to the system proposed by Gimenez-Conti and colleagues (2001).

RESULTS

Figure 1.1A depicts a “normal” X. nezahualcoyotl male with the Ss M pattern and typical micromelanophore patterning along the sword. Case A was noticed to be different from other conspecific males on 15 April 2005, when the entire sword was black because the two marginal stripes had merged. This male (4 August 2005) showed pigmentation progressing slightly into the caudal peduncle (Figure 1.1B). There appeared to be relatively little enhancement of pigmentation between 15 April 2005 and 4 August 2005. The superficial expression of melanin increased more rapidly between 4 August 2005 and 12 October 2005. The expression of melanin increased anteriorly and dorsally on this individual (Figure 1.1C, Figure 1.1D).

Case B has a different point of origin for the melanoma (Figure 1.2). Rather than originating from the sword stripes, in this individual the spotted-side macromelanophore pattern served as the cellular precursor for melanoma formation. The spotted side pattern usually consists of numerous individual medium to large size black spots, but in case B melanization increased until individual spots, especially towards the middle of the flank, were not easily distinguishable. This male was euthanized for histological evaluation, while the dorsal and ventral sword stripes were still clearly distinguishable (Figure 1.2).
There was a 113% increase in pigmented area of the sword stripes in case A between 4 August 2005 and 12 October 2005. The rate of melanin progression did show some variation, which was most pronounced early in the sequence. For example, from 4 August to 21 August 2005 (17 days) there was a 4% increase in melanin enhancement area, but from 21 August to September 7 2005 (17 days) there was ~35 % increase in the area of melanin (Table 1.1).

In case A, melanomas were found in a variety of locations in the fish (Figure 1.3, Figure 1.4, Figure 1.5). Depending upon the location examined, lesions found in the skin and underlying muscle ranged from precancerous melanosis of the skin to melanocytic melanoma of the skin and underlying muscle. Spindle cell type melanoma also occurred in lipid tissue between the hypaxial muscle posterior to the coelomic cavity (Figure 1.3). The most significant lesions observed were melanophorus-macromelanophorus polymorphic melanomas, which are mixed tumours containing an assortment of different cells: melanocytes, epithelioid-like cells, melanophores, and macromelanophores (Gimenez-Conti et al. 2001). These malignant lesions were observed in the region of the caudal peduncle and caudal fin where much of the subcutaneous tissues was invaded and replaced by the neoplasia, (Figure 1.4, Figure 1.5).

Lesions observed in case B were less severe than those in case A. Spindle cell type melanomas were found in the epidermis, dermis and underlying muscle in several locations on the fish (Figure 1.6). Spindle cell type melanoma was also found in lipid tissue between the hypaxial muscle posterior to the coelomic cavity (Figure 1.7).
DISCUSSION

These two specimens are the first documented cases of invasive, proliferative growth of neoplasms in *X. nezahualcoyotl*, whether non-hybrid or hybrid in origin. In case A neoplastic progression originated from a micromelanophore pattern which has not been documented in any unmanipulated *Xiphophorus* species. Previous research indicates that *X. nezahualcoyotl* lacks the *Xmrk* allele associated with melanoma formation in this model (Weis and Schartl 1998). Although the confirmation of the *Xmrk* genotype in either specimen presented here has not yet been conducted, it is likely that *X. nezahualcoyotl* is polymorphic for the *Xmrk* allele. This is supported by the presence of the spotted-side M pattern in both individuals which previous research has shown must be present in order for the *Xmrk* gene to be present (Weis and Schartl 1998). The assertion that *X. nezahualcoyotl* is polymorphic for *Xmrk* is also based on the histological similarities of the lesions in these two cases when compared to other non-hybrid melanomas in species that have *Xmrk* (*X. cortezi* from Morris laboratory, Ohio University; Schartl et al. 1995). Numerous factors can contribute to melanoma formation, for example, mutation within the pigment gene itself or promoter elements, thus it is possible that the neoplasms observed in both cases were not due to the *Xmrk* melanoma model.

The specimen of *X. nezahualcoyotl* presented here as case A developed multiple types of lesions and more severe lesions than case B. The most remarkable lesion was found on the caudal peduncle and caudal fin and was characterized as a melanophorus-
macromelanophorus polymorphic melanoma. This is considered to be the most malignant type of lesion according to the method for categorizing melanoma in *Xiphophorus* proposed by Gimenez-Conti and colleagues (2001). This neoplasia was heterogeneous, with variable staining intensities that reflected the cells of different shapes and sizes that comprised the affected area. Spindle cell type melanomas were also observed in several regions of the body posterior to the coelomic cavity. This type of melanoma was observed not only in the epidermis and dermis but also deep within the hypaxial muscle and adipose tissue which on the surface lacked melanin. The sequence of digital images confirmed that superficial melanization did not increase in other parts of the body such as the vertical bars and reticulum, which is consistent with melanoma formation in the *Xmrk* melanoma model (i.e., melanoma progression from a single M pattern). In contrast to the *Xmrk* model, melanomas did not originate from the M pattern, spotted side (which was unchanged), but rather from the micromelanophore patterning of the dorsal and ventral sword stripes.

A single type of melanoma was observed in case B based upon the system of nomenclature provided by Gimenez-Conti and colleagues (2001). Spindle cell type melanoma was documented in the epidermis, dermis, adjacent to the muscle tissue immediately below the dermal layer, and in the adipose tissue between the hypaxial muscle. However, I did not observe spindle cell type melanomas deeper within the hypaxial muscle of case B as I did in case A. As a result, melanoma formation in case B was restricted to only those areas that were superficially observed as black heavily pigmented areas (Ss M pattern). Melanoma formation was not observed in the caudal peduncle or the caudal fin of case B.
The work of Schartl and colleagues (1995) with a non-hybrid mutant strain of Southern platyfish (Xiphophorus maculatus, Günther) provides intriguing parallels to the melanoma formation originating from the micromelanophore patterning in case A. The mutation of the striped (Sr) gene in X. maculatus results in enhanced expression (melanosis) of the macromelanophore pattern Sr (Anders et al. 1973). Sr" males that also have the micromelanophore pattern of Lc (lower comet) and/or Ab (anal fin black) develop severe malignant melanomas originating from these micromelanophore patterns if they are older than 9 months and of “high social rank” (Schartl et al. 1995). Sr" males that lack these micromelanophore patterns or nonmutant Sr males with these patterns, fail to develop severe melanomas. The androgen dependency of these abnormalities were confirmed by adding steroids to the aquaria water and Xmrk specific melanoma associated antigens indicated the over expression of Xmrk in the Lc and/or Ab patterns of abnormal individuals (Schartl et al. 1995). The Lc pattern (a single stripe extending along the ventral edge of the caudal fin) of X. maculatus is phenotypically similar to the micromelanophore sword stripe pattern in X. nezahualcoyotl although this relationship, if any, is unclear. Case A was a wild caught X. nezahualcoyotl and did not represent a mutant strain; however, it is interesting that it had a M pattern and developed a severe melanoma originating from a micromelanophore pattern similar to Lc of X. maculatus. In addition, the male was more than nine months old and had been kept in a tank for more than a year with partial water changes every month (~15-20%). Thus, it is possible there was a build up of androgen metabolites in the tank that could have been responsible for the tumour induction.
The two *X. nezahualcoyotl* presented here were the only two individuals to develop abnormalities in two collecting seasons at the El Salto River site (April 2004 and December 2005; approx. 80 adult individuals). However, one should be hesitant to classify these as isolated events within the species of *X. nezahualcoyotl*. The generalizations made about individual susceptibility within *X. variatus*, for example, was based on ten years of field collections and observations of laboratory raised fish (of which only eight individuals had abnormalities, five with melanosis and three with nodular melanomas; Schartl et al. 1995). Therefore, additional sampling of *X. nezahualcoyotl* at the El Salto River collection site, as well as others within their range would be helpful in providing more information and insight into the prevalence and potential etiology of the observed melanoma formation. Future research investigating the link between androgen levels and melanoma formation originating from micromelanophore patterning would be useful since micromelanophore patterns are found in more species of *Xiphophorus* than macromelanophore patterns.
LITERATURE CITED


Electronic reference
Table 1.1 Melanoma progression in case A. Comparisons of melanin pattern area associated with melanoma progression within case A during August to October of 2005. The approximate percent increase represents the area of pattern increase from one picture day to the next whereas parenthetical values represent the percent increase from the original estimated area of the pattern on August 4 2005.

<table>
<thead>
<tr>
<th>Picture Date</th>
<th>Time interval between photographs (days)</th>
<th>Calculated Area (mm²)</th>
<th>Approximate percent increase</th>
</tr>
</thead>
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<tr>
<td>August 4 2005</td>
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<td>64.5</td>
<td>N/A</td>
</tr>
<tr>
<td>August 21 2005</td>
<td>17</td>
<td>67.1</td>
<td>4.0% (4)</td>
</tr>
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<td>September 7 2005</td>
<td>17</td>
<td>90.3</td>
<td>34.6% (40)</td>
</tr>
<tr>
<td>September 23 2005</td>
<td>16</td>
<td>107.7</td>
<td>19.3% (67)</td>
</tr>
<tr>
<td>October 12 2005</td>
<td>19</td>
<td>137.4</td>
<td>27.6% (113)</td>
</tr>
</tbody>
</table>
Figure 1.1 Photographs of *X. nezahualcoyotl*. (A) *X. nezahualcoyotl* male with normal melanin pigmentation, note dorsal and ventral sword stripes. (B) Case A male photographed 4 August 2005. (C) Case A male photographed 7 September 2005, grey arrows indicate areas of neoplastic progression. (D) Case A male photographed 12 October 2005. Insert depicts nodular lesions when viewed from above, which were not present in the early stages of the progression (Panel B and C). Scale bar: 5 mm.
Figure 1.2 Photograph of case B, male *X. nezahualcoyotl*. The spotted-side macromelanophore pattern originally was visible as distinct spots (similar to those in Figure 1.1A, normal specimen). Scale bar: 5 mm.
Figure 1.3 Case A body cross section. Cross section of the body of *X. nezahualcoyotl* (case A) in a region posterior to the coelomic cavity. Note presence of melanoma (M1) within the adipose tissue between the hypaxial muscle as well as adjacent to the muscle fibres deeper within the hypaxial muscle (M2).
Figure 1.4 Case A cross section caudal fin. Cross section of the caudal fin of *X. nezahualcoyotl* (case A) with melanophorus-macromelanophorus polymorphic melanoma. The width of the caudal fin has been expanded extensively by the mass of the tumour.
Figure 1.5 Magnification of box in Figure 1.4. High magnification of box in Figure 1.4 (case A), showing extensive invasion and replacement of subcutaneous regions of the caudal fin of *X. nezahualcoyotl* with the melanoma. Arrows indicate fin rays of the caudal fin.
Figure 1.6 Case B skin cross section. Cross section of skin of *X. nezahualcoyotl* (case B) with spindle cell type melanoma. Note the pronounced aggregation of melanocytes (M1) between and under the scales (S). One focus of macromelanophores (M2) can be seen below the dermis and adjacent to the muscle tissue. The detachment of the epidermis (E) from underlying tissue is an artifact.
Figure 1.7 Case B body cross section. Cross section of the body of *X. nezahualcoyotl* (case B) in a region posterior to the coelomic cavity. Note presence of spindle cell type melanoma (M) within the adipose tissue between the hypaxial muscle.
CHAPTER 2

XIPHOPHORUS MELANOMA RECEPTOR KINASE (XMRK) ONCOGENE IS CORRELATED WITH INCREASED MALE AGGRESSION IN XIPHOPHORUS CORTEZI
ABSTRACT

In *Xiphophorus* fishes, certain macromelanophore patterns are capable of forming malignant melanomas, which result from the overexpression of the *Xmrk* oncogene within the Ras/Raf/MAPK signaling pathway. How this oncogene has remained functional in an extremely unstable genomic region for millions of years is unknown. *Xiphophorus* males are more susceptible to melanomas, and have been noted to occur most frequently in *X. cortezi* males of high social rank. Despite this, the relationship between aggression and the *Xmrk* oncogene has not been investigated. The results of this study found that the spotted caudal (Sc) macromelanophore pattern in male *X. cortezi*, from which non-hybrid malignant melanomas can originate, is correlated with increased aggression in mirror image trials. Furthermore, *X. cortezi* males with the *Xmrk* oncogene (regardless of the presence of Sc) bite more and perform more agonistic displays than *Xmrk* deficient males. Male aggressive response decreases when viewing their Sc image as compared to their non-Sc image, demonstrating that the Sc phenotype is a signal used in male agonistic encounters within *X. cortezi*. The results of this study indicate that intrasexual selection is a potentially important component in the continued evolutionary maintenance of a functional oncogene within *Xiphophorus*.
INTRODUCTION

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 status in social arenas is well documented within the literature from a wide variety of taxa (Primates: Gerald 2001; Setchell and Wickings 2005; Birds: Rohwer 1977; Møller 1987 Lizards: Thompson and Moore 1991). Frequently, the expression of such traits directly covaries with testosterone levels (Rand 1992; Sinervo et al. 2000; Setchell and 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 and Metcalfe 2001; mates: Morris et al. 1992; Rantala and Kortet 2004).

_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. _in press_; sword size: Basolo 1990; Benson and Basolo 2006). Several behavioral studies have highlighted the specific importance of melanin patterns in sexual selection (Morris 1998; Basolo and Trainor 2002; Morris et al. 2003; Moretz and Morris 2006). For example, the melanin based vertical body bars have been shown to be attractive to females and also
function to deter rival males (Morris et al. 1995). However, the vast majority of these studies have focused on melanin patterns comprised of micromelanophores, which are smaller (up to 100 μM) and relatively more evenly spaced pigment cells than macromelanophores that tend to overlap at their margins and attain a much larger size (300-500 μM; Weis and Schartl 1998). This lack of behavioral studies investigating macromelanophore patterns is surprising because these patterns in numerous \textit{Xiphophorus} species are correlated with the presence of a functional oncogene (\textit{Xiphophorus} melanoma receptor kinase, \textit{Xmrk}) and serve as cellular progenitors of melanosis/melanomas within \textit{Xiphophorus} (Weis and Schartl 1998).

Although the discovery of melanoma formation within \textit{Xiphophorus} (Gordon 1927; Kosswig 1928) was initially induced through hybridization crosses (i.e., genetic release of autosomal suppressor gene; Meierjohann et al. 2004), it is now clear that certain \textit{Xiphophorus} species are susceptible to spontaneous melanomas in the absence of hybridization (Kallman 1971; Borowsky 1973; Chapter 1). Under both circumstances, the pathway leading to melanomas results from the overexpression of the \textit{Xiphophorus} melanoma receptor kinase gene (\textit{Xmrk}, oncogene) within the Ras/Raf/MAPK signaling cascade with subsequent neoplastic progression usually initiating from species-specific macromelanophore patterns (Schartl et al. 1995; Meierjohann and Schartl 2006). Macromelanophore patterns have been suggested to play a critical role in maintaining the \textit{Xmrk} oncogene (Weis and Schartl 1998; Meierjohann and Schartl 2006; see Chapter 3) because \textit{Xmrk} is only found in \textit{Xiphophorus} species with these patterns. Given the
deleterious nature of *Xmrk* it is not known how the oncogene has been maintained in several *Xiphophorus* species for millions of years (Meierjohann and Schartl 2006).

The northern swordtail *Xiphophorus cortezi* is polymorphic for the *Xmrk* oncogene. *Xmrk* is found on both the X and Y chromosomes and is an essential component for the expression of a spotted caudal (Sc) macromelanophore pattern (Schartl et al. 1995; Weis and Schartl 1998). 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 (Figure 2.1; Kallman 1971; Schartl et al. 1995). The degree of Sc expression (i.e., pattern size) varies dramatically both within and across *X. cortezi* populations (Figure 2.1; Kallman 1971; Fernandez unpublished data). The frequency of the Sc phenotype also varies considerably across *X. cortezi* populations (Table 2.3; Kallman 1971). All individuals with the Sc phenotype have *Xmrk* (Schartl et al. 95; Weis and Schartl 1998); however, due to incomplete penetrance of Sc, individuals that lack phenotypic expression of Sc can have the *Xmrk* genotype. Non-hybrid malignant melanomas originate from the Sc pattern and are deleterious, progressively destroying muscle tissue and ultimately impairing swimming ability (Kallman 1971; Schartl et al. 1995). Interestingly, unlike *Xiphophorus variatus* in which the occurrence of melanomas is equal between the sexes, the incidence of melanoma formation is higher in *X. cortezi* males than females (Schartl et al. 1995). In addition, Schartl and colleagues (1995) state melanomas in *X. cortezi* occur most frequently in ‘sexually active males of high social rank’ although they did not elaborate. This suggests that there may be a relationship between aggression and the *Xmrk*
oncogene. In fact, exogenously applied testosterone, which is positively correlated with aggression in *Xiphophorus helleri* (Hannes 1984), not only increases melanoma malignancy but also is sufficient to induce melanoma formation in *Xiphophorus maculatus* with macromelanophore patterning (i.e., Xmrk individuals; Schartl et al. 1981; Schartl et al. 1982; Schartl et al. 1995).

Although testosterone’s ability to influence melanoma formation is apparent, no study has established a correlation between Xmrk and male aggression. In a recent study, Franck and colleagues (2001) found females preferred *X. helleri* males with the polymorphic macromelanophore pattern dabbed (Db) although Db patterned males were not more successful in dyadic contests than non-Db males. However, *X. helleri* is the only species with macromelanophore patterning that has repeatedly been shown to lack the Xmrk oncogene (Weis and Schartl 1998; Meierjohann et al. 2004). In order to properly investigate a potential link between sexual selection and the evolutionary maintenance of Xmrk within *Xiphophorus*, studies need to be conducted on a species that has retained the Xmrk genotype. The purpose of this study was to examine the potential benefits, if any, the Xmrk genotype (and the associated Sc macromelanophore pattern) might confer to males via intrasexual selection in *Xiphophorus cortezi*. I use male mirror image stimulation tests to specifically address: 1) Are Sc pattern males more aggressive than non-Sc pattern males; 2) Are males with the Xmrk genotype more aggressive than wild type males without the Xmrk genotype; and 3) Does male aggressive response differ based upon the presence of the Sc phenotype in the mirror image?
METHODS

Specimen collection and housing

*Xiphophorus cortezi* males used in this study were collected from five natural populations within the Río Pánuco basin: Arroyo Tanute N 21 39 123, W 99 02 127; Arroyo Chalpuhuacanita N 21 12 364, W 98 40 153; Rio San Martín 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 Potosí provinces; Mexico). Collection sites were selected to maximize sampling of *X. cortezi* populations based upon a phylogenetic reconstruction of *X. cortezi* haplotypes (Gutierrez-Rodriguez et al. 2007). All males were collected as adults during two field seasons: December 2005 (Conchita, n = 36, Mean SL = 39.3 mm, s. d. = 4.4; Tanute, n = 27, Mean SL = 35.0 mm, s. d. = 4.2) and April 2006 (Chalpuhuacanita, n = 23, Mean SL = 38.7 mm, s. d. = 3.9; San Martín, n = 37, Mean SL = 37.7 mm, s. d. = 3.5; Tecolutlo, n = 16, Mean SL = 39.3 mm, s. d. = 3.2). Standard length (SL) was defined as distance from the tip of the snout to the base of the caudal peduncle. Upon return to the United States, males from each population were housed in communal tanks with females from the same locale. 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).
Male mirror image stimulation

Standard mirror image stimulation (MIS) tests were used to determine if the expression of spotted caudal (Sc) affects male aggression and to investigate the potential function of the Sc pattern in aggressive encounters. Aggression associated with the expression of Sc was determined by comparing the aggressive interactions of naturally occurring Sc males with those of painted Sc males (males did not have natural Sc expression). Therefore, both males in this comparison were seeing the same image (Sc patterned male), and differences in their aggression levels were quantified. In the second comparison, male response to the Sc pattern was assessed within males that naturally lacked the Sc phenotype by comparing their aggressive responses to both their artificially painted Sc and their natural non-Sc image. 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 numerous Xiphophorus species including X. cortezi (Franck et al. 1985; Moretz and Morris 2003).

Temporary Sc pattern was applied to non-Sc males using Dr. Naylor’s Blue-Kote antiseptic dye (H. W. Naylor Co., Inc., Morris, NY; Hoefler and Morris 1999). The painted Sc pattern represented an approximate average size Sc pattern for that particular population. In the case of Tecolutlo, in which all individuals surveyed and collected lacked Sc (see Chapter 4), a representative Sc phenotype was painted based upon the other four populations. Natural Sc males and non-Sc males in trials testing response to non-Sc image 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 otherwise alters their behaviors (Hoefler and Morris 1999). The two tests conducted on non-Sc males were performed at least two weeks apart in order to reduce the influence of the first trial experience. Two weeks is sufficient to reduce the effects of prior encounters (Moretz and Morris 2003). Treatment order was randomized.

Experimental tanks were 19L aquaria with a line 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, males were measured (SL) and information was collected about the individual’s phenotype including the presence of Sc and vertical body bars which have 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. 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 (water and dye). The five minute trial period began as soon as the individual entered the interaction zone and directly faced his image. Interaction time was defined and recorded 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 and Causey 1989); vertical headstand, head tilts downward until body is at ~45° angle with the substrate (Lyons and Morris in press). These display types are common in actual confrontations in *X. cortezi* (Moretz and Morris 2003). Finally, I recorded the number of bites directed at the mirror image during the trial period.

**DNA analysis**

DNA was not collected in five of the 27 males from Tanute and one of the 16 males from Tecolutlo, therefore, these males were removed from all analyses involving male *Xmrk* genotype. Males were fin clipped after their last MIS test and DNA was extracted using DNeasy® tissue kit (Qiagen Inc.) following the manufacturer’s instructions. Total elution volume was 100 µl. The presence of an oncogenic copy of the *EGFR* was determined by cross-referencing the polymerase chain reaction (PCR) products of two primers sets developed in the laboratory. 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 (EGR-B). The following primer set was used to screen for the presence of the *Xmrk* genotype: “Montoncoup” sense primer 5´- GGTCATAAATCACTCATCCAT located in the promoter region at nt 21-43 (nt numbering according to AY298858; Volff et al. 2003) and “Dwnmont2” antisense primer 5´- ACAAGTTTGAGAAATAACCTGAACTC 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’- GATGTTACTTTAGTCTGTGAGTC 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) yields a total of three bands: proto-oncogene band at ~750 bp and two oncogene bands, one at ~950 bp and one at ~1100 bp. 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).

**Statistical analysis**

With the exception of Chalpuhuacanita, some males from each population did not respond to the placement of the mirror (Table 2.1). Male ‘response’ was defined as a male entering the interaction zone within thirty minutes of mirror placement (‘no response’ failure to enter within thirty minutes). All 23 males from Chalpuhuacanita responded to the placement of the mirror. Initially, I was interested in conducting a log linear analysis to examine if male response (or the lack of response) was influenced by the male’s native population, their Xmrk genotype, and their vertical body bar phenotype. Log linear analysis determines if relationships exist among categorical variables and allows the investigator to examine all levels of main effects and interaction effects. However, because five populations of *X. cortezi* were collected, including population as a variable in the log linear model dramatically increased the dimensionality of the contingency table (8 cells to 40 cells) and subsequently decreased the count data in each cell (Table 2.1). Because 12 cells had zero counts and several more had limited counts, creating a model with all four factors (i.e., population, Xmrk genotype, Vertical bar...
phenotype, and response) was not possible. As a result, I ran two types of analyses to determine the influence of population, Xmrk, and vertical bars on male response to mirror image stimulation. First, I performed a cross tabulation between population and male response to determine if male response was influenced by their native population. Second, I built a log linear model with the factors male response, male Xmrk genotype, and male vertical bar phenotype. This second analysis examined the possible influences of Xmrk and vertical body bars on male response to mirror stimulation, including the possibility of interaction effects (e.g., males with Xmrk and vertical body bars).

For the males that did respond to mirror image stimulation, the possible influence of the male’s resident population on the four recorded aggression variables was assessed using Kruskal-Wallis ANOVAs. I used a nonparametric Mann Whitney U test to compare the mean aggression levels of Sc and non-Sc males (referred to hereafter as ‘aggression comparison’). Wilcoxon signed ranks test was calculated to determine if males responded differently to their Sc and non-Sc images (referred hereafter as ‘male response to Sc’).

**RESULTS**

The cross-tabulation analysis revealed that the population a male was from influenced whether or not that male responded in the mirror image trials (Table 2.1; Chi Square test: N = 133, $X^2_{4} = 21.4$, $P < 0.001$). Subsequently, Fisher exact tests indicated that males from Chalpuhuacanita were more likely to respond to mirror image stimulation
than males from the other four populations (Fisher Exact test: compared against Conchita, $P = 0.04$; Tanute, $P < 0.001$; Tecolutlo, $P = 0.05$; and San Martín, $P = 0.002$).

In addition, males from Conchita were more likely to respond than males from Tanute (Fisher’s exact test: $P = 0.016$). However, the log linear model found there was no relationship between male \( Xmrk \) genotype and male vertical bar phenotype, and whether or not that male responded to the mirror image stimulation (Log linear model: Partial $X^2 = 1.784$, $P = 0.775$). In addition, there was no difference in the size of males who responded to MIS tests (mean SL of males who responded = 38.1 mm, s.d. = 4.2, mean SL of males who did not respond = 37.2 mm, s.d. = 4.0; two-tailed $t$-test: $t = 1.09$, $P = 0.28$). Because males who did not respond to the placement mirror ($N = 31$) could not be predicted by any of the variables of interest, these males were excluded from all male aggression analyses.

The influence of population on the specific aggressive behaviors of males who responded to mirror image stimulation ($N = 102$) was analyzed separately. In the case of the aggression comparison, I conducted Kruskal-Wallis ANOVAs with population as the main effect for the recorded data of both the natural Sc males ($N = 20$) and the painted Sc males ($N = 48$). There was no effect of population on any of the four dependent variables recorded for either the natural Sc males (Kruskal-Wallis ANOVA: headstands: $X^2_2 = 2.832$, $P = 0.243$; lateral: $X^2_2 = 2.809$, $P = 0.245$; bites: $X^2_2 = 1.49$, $P = 0.475$; interaction time: $X^2_2 = 3.817$, $P = 0.148$) or the painted Sc males (Kruskal-Wallis ANOVA: headstands: $X^2_2 = 4.764$, $P = 0.092$; lateral: $X^2_2 = 0.079$, $P = 0.961$; bites: $X^2_2 = 1.177$, $P = 0.555$; interaction time: $X^2_2 = 0.562$, $P = 0.755$). Thus, the amount of aggression for each
treatment was consistent across populations of the males that responded to the MIS experimental design. Because each male was tested twice in the male response to Sc comparison, 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. Once again, males from different populations did not differ in their aggressive responses for all four variables measured (Kruskal-Wallis ANOVA: headstands: $X^2_4 = 3.799, P = 0.434$; lateral: $X^2_4 = 0.769, P = 0.943$; bites: $X^2_4 = 7.961, P = 0.093$; interaction time: $X^2_4 = 4.873, P = 0.301$). Therefore, because male aggression did not vary across the five populations sampled, I pooled the male data for each of the four variables in both the aggression and male response to Sc comparisons.

Spotted caudal males spent significantly more time interacting with their mirror image than non-Sc males painted with the Sc pattern (Table 2.2; Mann-Whitney U test: $Z = -2.341, P = 0.019$). Males with naturally occurring Sc also bite more at their image than painted Sc males (Figure 2.2A; mean bites natural Sc males = 7.3, mean bites painted Sc males = 3.06; Mann-Whitney U test: $Z = -2.095, P = 0.036$). There was no difference in the number of displays performed by Sc males and painted Sc males (mean headstands natural Sc males = 1.2, mean headstands painted Sc males = 1.77; Mann-Whitney U test: $Z = -0.242, P = 0.808$; mean laterals natural Sc males = 1.15, mean laterals painted Sc males = 0.71; Mann-Whitney U test: $Z = -1.523, P = 0.128$).

However, because the Sc pattern has incomplete penetrance (Kallman 1971), X. cortezi males without phenotypic expression of spotted caudal can have the associated Xmrk oncogene (Table 2.3). Thus, it is possible for painted Sc males in this analysis,
which lacked natural Sc expression, to have the \textit{Xmrk} genotype. Because I was also interested in determining if \textit{Xmrk} was associated with increased male aggression, males were grouped in the aggression comparison by their \textit{Xmrk} genotype as well. As expected, \textit{Xmrk} males spent more time interacting with their image than \textit{Xmrk} deficient males (Table 2.2; Mann-Whitney U test: $Z = -3.06, P = 0.002$). Males with the \textit{Xmrk} genotype also bite more (Figure 2.2A; mean bites \textit{Xmrk} males = 6.23, mean bites no \textit{Xmrk} males = 1.58; Mann-Whitney U test: $Z = -2.264, P = 0.024$) and did more lateral displays (Figure 2.2B; mean laterals \textit{Xmrk} males = 1.08, mean laterals no \textit{Xmrk} males = 0.54; Mann-Whitney U test: $Z = -2.146, P = 0.032$) at their image than \textit{Xmrk} deficient males. There was no difference in the number of headstands performed by \textit{Xmrk} and \textit{Xmrk} deficient males (mean headstands \textit{Xmrk} males = 2.03, mean headstands no \textit{Xmrk} males = 1.17; Mann-Whitney U test: $Z = -1.351, P = 0.177$). There was no significance difference in the aggression levels of \textit{Xmrk} males with the Sc pattern and \textit{Xmrk} males who did not naturally express Sc but were painted with Sc (Mann-Whitney U test: headstands: $Z = -0.903, P = 0.367$; laterals: $Z = -0.362, P = 0.717$; bites: $Z = -0.887, P = 0.398$; interaction time: $Z = -0.746, P = 0.455$). Lastly, the relationship we detected between male aggression and \textit{Xmrk} (and Sc) cannot be explained by the presence or absence of vertical body bars in \textit{X. cortezi} males, which has previously been shown to influence aggression (Moretz and Morris 2003; Moretz 2005). Males with bars did interact more than barless males (Mann-Whitney U test: $Z = -2.290, P = 0.022$) however there was no significance difference in the three aggression variables measured between the two morphs (Mann-Whitney U test: headstands: $Z = -1.364, P = 0.172$; laterals: $Z = -0.147$,
In the male response to Sc comparison, males bite more at their non-Sc image than at their Sc image (Figure 2.3A; mean bites towards non-Sc image = 11.46, mean bites towards Sc image = 5.46; Wilcoxon signed ranks test: Z = −3.368, P = 0.001). Males also performed more lateral displays towards their non-Sc image than their Sc image (Figure 2.3B; mean laterals towards non-Sc image = 3.55, mean laterals towards Sc image = 2.05; Wilcoxon signed ranks test: Z = −2.544, P = 0.011). Male response to Sc was not different for the number of headstand displays performed (mean headstands towards Sc image = 3.18, mean headstands towards non-Sc image = 2.48; Wilcoxon signed ranks test: Z = −1.080, P = 0.28) or interaction time (Table 2.2; Wilcoxon signed ranks test: Z = −1.767, P = 0.077).

**DISCUSSION**

This study found that *Xiphophorus cortezi* males with the Sc pattern and therefore the *Xmrk* oncogene bit more and performed more agonistic displays at their image than males without the Sc phenotype. In addition, males with *Xmrk*, regardless of whether they expressed the Sc pattern, were also more aggressive than *Xmrk* deficient males. This implies that the *Xmrk* oncogene is underlying the increased aggression in males and not the expression of the Sc phenotype itself. The differences in male aggressive response towards the Sc pattern indicate that the Sc phenotype (Figure 2.3) does function as a
signal in *X. cortezi* male agonistic encounters. *X. cortezi* males without Sc, regardless of their *Xmrk* genotype, performed significantly fewer lateral displays and bites towards their Sc mirror image when compared to their non-Sc image, which would be expected given the increased aggression associated with Sc patterned males. This result supports the idea that Sc alone (males served as their own controls) is sufficient to convey information about aggression and therefore could play an important function in determining the outcome and duration of male encounters. Because Sc is associated with increased aggression in males, and males differentially respond to this signal, the Sc phenotype in *X. cortezi* might serve as a badge of status according to the status-signaling hypothesis.

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 and Camerino 1998). Although it is clear that Sc varies dramatically in size not only within but also between populations (Figure 2.1; Kallman 1971), the relationship between the extent of Sc expression and dominance and/or aggression has not been formally established. However, the size of Sc (and other macromelanophores within this system) is influenced by the overexpression of *Xmrk*, which increases the phenotypic expression of the associated macromelanophores (Chapter 3; Schartl et al. 1995; Weis and Schartl 1998; Meierjohann and Schartl 2006). Similarly, enhancements in the phenotypic expression of macromelanophore patterns can also result from exposing *Xmrk* individuals to elevated levels of testosterone (Schartl et al. 1981; Schartl et al. 1982;
Schartl et al. 1995), which is correlated with aggression levels in *Xiphophorus* (Hannes 1986). Hannes (1986) specifically demonstrated that the number of bites and sigmoid threat displays in agonistic encounters were positively correlated with the testosterone concentrations in blood and whole body tissues of male *X. helleri*. Therefore, it is not unreasonable to suggest a positive relationship exists between the increased aggression levels in *X. cortezi* males carrying Xmrk and the degree of Sc expression; however, future hormonal research needs to confirm this assumption within this species.

The use of male mirror image stimulation (as opposed to dyadic contests) to assess dominance has received some criticism because the outcomes are not always consistent (Meliska et al. 1980; Ruzzante 1992; although see Holtby 1992). For example, individual *Betta splendens* that differentially responded during mirror tests became indistinguishable in their aggression measures during dyadic contests (Meliska et al. 1980). However, 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. Previous work in *X. cortezi*, which focused on the vertical bar phenotype, found that males are consistent in aggression measures across dyadic contests and male mirror image stimulation (Moretz and Morris 2003; Moretz 2005). Specifically, barless *X. cortezi* males bite more during
MIS trials and male dyadic contests than barred males. More importantly for the implications of this study was the finding that _X. cortezi_ males that bite most frequently were the eventual winners of dyadic contests (i.e., dominant; Moretz 2005).

While collecting _X. cortezi_ from the field for the current study, juveniles were observed possessing the Sc pattern. Although, juveniles would not benefit from mating preferences for Sc (see Chapter 3, 4), there are several possible benefits a juvenile might receive from expressing Sc before sexual maturation. First, juvenile pigmentation is likely to assist in schooling behavior under turbid water conditions, which occur after periods of heavy rainfall, because this has been demonstrated in adult _X. helleri_ (Franck et al. 2001). Second, given our findings of decreased aggressive response to Sc, it is possible that juveniles with Sc might experience reduced aggression from adults in the population. Lastly, the increased aggression associated with Sc and the _Xmrk_ oncogene could benefit _X. cortezi_ juveniles by allowing them greater access to food resources. Juvenile aggression is not uncommon in poeciliids and has even been documented in three day old guppies (Gorlick 1976). Greater access to food resources could be important not only in influencing age at maturation (Metcalfé and Monaghan 2003), but also because juvenile food acquisition has been shown to be a critical factor in determining the outcome of future male-male agonistic encounters in _Xiphophorus helleri_ (Royle et al. 2005).

_Xiphophorus_ is well known for their flexibility in the age at maturation (pituitary alleles: Kallman 1973; rearing environment: Morris and Ryan 1990). In fact, a recent study found that age at sexual maturation in _X. helleri_ is contingent on the level of male
ornamentation within the rearing environment; females mature earlier and males delay maturation when viewing large-sworded males as compared to short-sworded males (Walling et al. 2007). Such studies highlight the importance of visual cues in *Xiphophorus* ontogeny and raise many questions concerning the developmental plasticity of patterns used in sexual selection within *Xiphophorus*. Although virtually nothing is known about the development plasticity of macromelanophore patterns, the social environment during maturation likely influences the expression of these patterns. This is because the expression of macromelanophore patterns, like other secondary sexual characteristics within *Xiphophorus* (Zauner et al. 2003), is androgen dependent (Schartl et al. 1981; Schartl et al. 1982; Schartl et al. 1995). Given that the gonads of *Xiphophorus* fry are capable of synthesizing these hormones (Schreibman et al. 1982), the developmental timing and expression of macromelanophore patterning is expected to be plastic similar to other androgen dependent traits in *Xiphophorus* (Zauner et al. 2003 and references therein). The effect that social sexual experience has on aggression levels in *X. cortezi*, the penetrance of *Sc* in a population, and the degree of *Sc* expression is unknown and warrants investigation. Similarly, stochastic environmental variables (e.g., predation intensity, water turbidity) should also favor flexibility in the expression of *Sc* within *X. cortezi* especially given that those who do not express the pattern would retain the benefits of increased aggression, which this study found to be an intrinsic quality associated with the *Xmrk* oncogene.

The location of the *Xmrk* oncogene on the sex chromosomes is in the proximity of a number of genes important in sexual selection, including the master sex determining
gene (SD), the pituitary locus (P alleles), and the red-yellow locus (RY; Meierjohann et al. 2004). For example, the RY locus 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 and Trainor 2002; Franck et al. 2003). The close proximity of such loci to the *Xmrk* oncogene certainly presents the possibility for genetic hitch-hiking (Maynard Smith and Haigh 1974; Franck et al. 2001) and even has the potential to explain the continued evolutionary maintenance of *Xmrk*. In addition, attributes associated with *Xmrk* (e.g., aggression) should become correlated with physical traits such as body size and coloration encoded by these adjacent loci via genetic linkage. For example, a study of aggression in red and black color morph hybrids (*X. helleri*-*X. maculatus*) found that red morphs were consistently more aggressive than black morphs (Heuts and Nijman 1998). The authors suggest that in attempting to create different color morphs, breeders inadvertently selected for ‘color-linked genes’ that produced the observed differences in agonistic behavior (Heuts and Nijman 1998). Here I suggest that *Xmrk* is the color-linked gene, the presence of which could explain the increased aggression of the red morph.

Here I show that *X. cortezi* males with Sc and/or *Xmrk* are more aggressive than wildtype individuals, and that males perceive the Sc phenotype as a signal in aggressive encounters. Recently, it was demonstrated that *X. cortezi* females prefer Sc patterned males to non-Sc males, and that females prefer larger Sc patterns in males to size matched males with smaller Sc patterns (see Chapter 3). Collectively, these findings demonstrate that the Sc macromelanophore pattern in *X. cortezi* is a visual signal used in
sexual selection, comparable to certain micromelanophore patterns within *Xiphophorus*. An important distinction however is that only macromelanophore patterns are closely associated with the *Xmrk* oncogene and can serve as site of melanoma formation (although see Chapter 1). It is suggested that sexual selection for these associated macromelanophore patterns plays an important role in the continued evolutionary maintenance of *Xmrk* within *Xiphophorus*. Within *X. cortezi*, the early non-lethal stages of neoplastic progression, which would increase the Sc visual signal, are selected for through female mate choice (see Chapters 3, 4) and should be advantageous in male-male competition as well. Sexual selection for Sc during these non-lethal stages of the disease is likely strong enough to supersede the deleterious nature of *Xmrk*, ultimately resulting in its evolutionary conservation over time.
LITERATURE CITED


Table 2.1  Contingency table of *X. cortezi* male response to mirror image trials. This table represents all genotyped males (N = 133), dividing them according to whether or not they responded to mirror stimulation, their *Xmrk* genotype, their vertical bar phenotype, and their native population. The male aggression comparisons (i.e., aggression and male response to Sc) were conducted on only those individuals below the dashed line who responded to the placement of the mirror on their resident tank.

<table>
<thead>
<tr>
<th>Response</th>
<th>Xmrk</th>
<th>Bars</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chalpu.</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>3</td>
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<tr>
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<td>11</td>
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</tr>
<tr>
<td></td>
<td>Yes</td>
<td>6</td>
<td>14</td>
</tr>
</tbody>
</table>
Table 2.2  Male interaction times. Mean amount of time (sec) males spent interacting with their mirror image in MIS trials. In the aggression comparison, males were either grouped by treatment (natural Sc/painted Sc) or by $Xmrk$ genotype ($Xmrk$/No $Xmrk$). SEM are in parentheses.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Male condition</th>
<th>Mean interaction time</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>treatment</td>
<td>natural Sc</td>
<td>219.7 (23.1)</td>
<td>$p = 0.019$</td>
</tr>
<tr>
<td></td>
<td>painted Sc</td>
<td>141.0 (18.0)</td>
<td></td>
</tr>
<tr>
<td>$Xmrk$</td>
<td>$Xmrk$</td>
<td>198.5 (19.1)</td>
<td>$p = 0.002$</td>
</tr>
<tr>
<td></td>
<td>No $Xmrk$</td>
<td>110.8 (21.6)</td>
<td></td>
</tr>
<tr>
<td>Male response to Sc</td>
<td>no Sc</td>
<td>188.8 (12.4)</td>
<td>$p = 0.077$</td>
</tr>
<tr>
<td></td>
<td>painted Sc</td>
<td>162.5 (12.7)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3 Number of *X. cortezi* in MIS trials. Total number of *X. cortezi* males included in male MIS analyses from each of the five populations. For each population, the frequency of the spotted caudal (Sc) phenotype and the *Xmrk* genotype of males are listed. Note the increase in the frequency of *Xmrk* males compared to Sc males for each population due to the incomplete penetrance of the Sc pattern.

<table>
<thead>
<tr>
<th>Population</th>
<th>Total</th>
<th>Sc males</th>
<th>Non-Sc males</th>
<th><em>Xmrk</em></th>
<th>No <em>Xmrk</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chalpuhuacanita</td>
<td>23</td>
<td>7</td>
<td>16</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>Conchita</td>
<td>30</td>
<td>10</td>
<td>20</td>
<td>23</td>
<td>7</td>
</tr>
<tr>
<td>Tanute</td>
<td>15</td>
<td>3</td>
<td>12</td>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tecolutlo</td>
<td>13</td>
<td>0</td>
<td>13</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>San Martin</td>
<td>26</td>
<td>0</td>
<td>26</td>
<td>1</td>
<td>25</td>
</tr>
</tbody>
</table>

<sup>a</sup> No DNA for four males  
<sup>b</sup> No DNA for one male
Figure 2.1 Variation in Sc phenotype. These *X. cortezi* males (Panels A-D) were all collected on the same day from the Conchita collection site (San Luis Potosí, Mexico). This amount of variation in pattern size and saturation is typical across *X. cortezi* populations. Scale bar: 5 mm.
Figure 2.2  Male aggression associated with Sc and Xmrk. Mean number of bites (A) and lateral displays (B) by X. cortezi males during the five minute MIS trials. In this comparison all males saw their mirror image with the Sc phenotype (non-Sc males had Sc painted on). For each dependent variable, males were grouped according to their natural Sc phenotype (Sc/ no Sc) and their Xmrk genotype (Xmrk/No Xmrk). Bars represent the mean ± SEM.

(A) $p = 0.036$  

(B) $p = 0.128$  

$Xmrk$ genotype (Xmrk/No Xmrk). Bars represent the mean ± SEM.
Figure 2.3 Male response to Sc pattern. Mean number of bites (A) and lateral displays (B) by *X. cortesi* 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 ± SEM.

(A) and (B)
CHAPTER 3

MATE CHOICE FOR MORE MELANIN AS A MECHANISM TO MAINTAIN A FUNCTIONAL ONCOGENE
ABSTRACT

The mechanisms by which cancer evolves and persists in natural systems have been difficult to ascertain. In the *Xiphophorus* melanoma model, a functional oncogene (*Xiphophorus* melanoma receptor kinase, *Xmrk*) has been maintained for several million years despite being deleterious and in an extremely unstable genomic region. Melanomas in *Xiphophorus* fishes (platyfishes and swordtails) have been investigated since the 1920s, and yet positive selection that could explain the maintenance of *Xmrk* has not been found. Here I show that *Xiphophorus cortezi* females from two populations prefer males with the spotted caudal (Sc) melanin pattern, which is associated with the presence of the *Xmrk* oncogene and serves as the site of melanoma formation within this species. Moreover, *X. cortezi* females prefer males with an enhanced Sc to males with a reduced Sc pattern. RT-PCR analysis confirms tissue specific *Xmrk* expression within the Sc pattern in *X. cortezi*. Because of the association of *Xmrk* with the Sc pigment pattern, and the fact that melanoma formation augments this visual signal, sexual selection appears to be maintaining this oncogene due to a mating preference for Sc as well as the exaggeration of this male trait. At the individual level, decreases in viability and fecundity due to *Xmrk* and subsequent melanoma formation may be mitigated via increases in mate acquisition. At the population level, maintenance of this oncogene appears to be under frequency dependent selection, as I detected female preference for males without Sc in a third population that had higher frequencies of Sc in females.
The evolutionary origin of cancer may be an inevitable outcome of multicellularity, cell replacement and genetic changes (Graham 1992; Greaves 2000). Yet this does not explain how cancer can (and does) persist over evolutionary time. Though seemingly counterintuitive, several cancer cell lineages recently have been shown at the genetic level to be under positive selection (for review see Crespi and Summers 2006). For example, strong directional selection has been detected in the coding regions flanking the mammalian testis determining gene ($S_{ry}$) in humans and primates (Whitfield et al. 1993) despite $S_{ry}$ expression in human prostate cancer (Tricoli et al. 1993). However, the maintenance of such oncogenes is generally considered the byproduct of genomic conflict (Kleene 2005) and antagonistic coevolution (e.g., maternal-fetal interactions: Lala et al. 2002; Summers and Crespi 2005). One recent study has even implicated a possible trade-off involving sexual selection; a shorter CAG repeat region within the androgen receptor gene can increase fertility but may also increase an individual’s risk of developing prostate cancer (Summers and Crespi 2008). Genes with oncogenetic potential could benefit an individual if their expression is necessary for and/or enhances a phenotype under sexual selection. Pigment patterns function as sexually selected signals across extremely diverse taxa (Andersson 1994), with the most desirable mate often having the most pronounced visual trait (for review see Ryan and Keddy-Hector 1992). Sexual selection for enhanced pigment patterns would result in the increased propagation of pigment cells used in creating the trait. Because increasing the size of a pigment
pattern can also be characteristic of melanoma formation, I examined the possibility that sexual selection could contribute to the maintenance of a cancer-causing gene.

The study of hybrid crosses within *Xiphophorus* and the resulting melanomas led to the initial realization that cancers can have a heritable basis (Meierjohann and Schartl 2006). More recently, non-hybrid melanoma formation has also been confirmed in three species of *Xiphophorus* (Schartl et al. 1995; Chapter 1), including *X. cortezi*. In both hybrid and non-hybrid melanomas, overexpression of the *Xmrk* oncogene occurs within species-specific macromelanophore patterns (Schartl et al. 1995; Weis and Schartl 1998). *X. cortezi* is polymorphic for the macromelanophore pigment pattern spotted caudal (Sc) and the associated *Xmrk* oncogene (Kallman 1971; Schartl et al. 1995). *Xmrk* is an essential component for the expression of the Sc phenotype in *X. cortezi* (Schartl et al. 1995; Weis and Schartl 1998; Figure 3.1) and can be located on the X and/or Y chromosomes (Froschauer et al. 2002). Therefore, malignant melanoma formations originating from the Sc macromelanophore pattern have been documented in both sexes of *X. cortezi* (Kallman 1971; Schartl et al. 1995; Figure 3.2). All individuals with the Sc phenotype have at least one copy of *Xmrk*. However, due to the incomplete penetrance of Sc, individuals that lack phenotypic expression of Sc can also have one or two copies of *Xmrk* (Kallman 1971). Screening six populations of *X. cortezi* for the *Xmrk* genotype has confirmed previous results (Schartl et al. 1995; Weis and Schartl 1998) of the consistent association between Sc and *Xmrk* (*N* = 98 Sc individuals, *N* = 552 total *X. cortezi*; see Chapter 4).
Using standard dichotomous choice tests, I examined female mate preference for the macromelanophore pattern Sc in 3 populations (Cebolla, Tanute, Conchita) of X. cortezi. In the first experiment, I examined if there was a preference for larger Sc patterned males that could explain the correlation between Sc and the Xmrk oncogene, as the overexpression of Xmrk can enhance this pigment pattern (Schartl et al. 1995; Weis and Schartl 1998). I used time associating with a stimulus as a measure of mating preference. Association time has been shown to be indicative of mate preference in fishes such as the closely related swordtail X. nigrensis (correlated with male mating success in the field; Ryan et al. 1990; Morris et al. 1992) and Gambusia holbrooki (correlated with male mating success in the laboratory; Bisazza et al. 2001). In addition, a recent study conducted on X. nigrensis found that association time is a more consistent and repeatable estimate of female preference than other female behaviors (Cummings and Mollaghan 2006). In the second experiment, I examined the possibility that the frequency of the Sc phenotype in a population could influence the strength of preference for Sc given the increased costs associated with having two copies of the Xmrk oncogene (Kallman 1971; Schartl et al. 1998).

**METHODS**

*Specimen collection and housing*

All fish used in these experiments were collected from the following natural populations: Arroyo Tanute N 21 39 123, W 99 02 127; Arroyo Cebolla N 21 23 472, W
Representative samples were collected as adults during two field seasons: December 2005 (Conchita and Tanute) and January 2007 (Cebolla). Upon return to the United States, the fish were individually housed at Ohio University in 19 l tanks and visually isolated from one another for a minimum of 4 weeks prior to conducting female preferences tests. All fish were maintained under standard laboratory conditions throughout the experiments which included a 12L:12D cycle, daily feeding (Tetramin® flakes), and a constant temperature of 22° C.

**Male treatments**

Pairs of males were matched for standard length within ± 2 mm. Because *X. cortezi* females prefer males with symmetrical barring (Morris 1998; Morris and Casey 1998) and have polymorphic preferences for males with vertical bars (Morris et al. 2003), males were also paired according to their bar state (i.e., not differing by more than a total of 3 vertical body bars), bar symmetry and overall similarity in melanin pigment patterning. Paired males were always from the same population. All males used as stimulus males lacked the Sc pattern. Each experiment used seven pairs of stimuli males, with the exception of the experiment testing for preferences of large Sc-small Sc (Cebolla population), which used eight pairs. I randomized which pair of males was used with each female. I tested nineteen females from Tanute, twenty-one females from Cebolla, and twenty-seven females from Conchita. All females were wild caught.
In the investigation of female preferences for the presence of Sc phenotype, one male in each pair was randomly chosen to receive a painted Sc treatment of average size using the antiseptic dye Dr. Blue Kote (H. W. Naylor Co., Inc., Morris, NY; Hoefler and Morris 1999) while the other male received a mock water painting. The size of the Sc phenotype applied to males was consistent and was representative of the average Sc expression for the population being tested based on measurement of wild caught males from that site (see Figures 3.3 and 3.4 for photographs of Sc painted treatments). Nineteen females from the Cebolla population were tested for their preferences of enhanced Sc phenotype. Males in this experiment randomly received either a small Sc phenotype (~20th percentile of expression) or received a large Sc phenotype (~80th percentile), giving the female a choice between large Sc and small Sc. This experiment was conducted with females from the Cebolla population only and females were tested 5 weeks after I conducted the presence-absence Sc experiment.

In both experiments, each female was tested with the same pair of males twice; however, the treatment each male received was switched across test days. Switching males between treatments allowed me to control for any unforeseen behavioral or phenotypic differences between the pairs of stimulus males. Within 3-4 hours the antiseptic dye fades which makes it possible to switch males between treatments on test days. I randomized which pair of males was tested with each female. All observation trials, including the painting of treatments in these trials, were conducted by one person (A.A.F.) to reduce variation in the painting of males across females.
Female preference tests

I used a standard dichotomous choice test design (for tank description see Morris et al. 2001). A twin light fixture with Vitalite® fluorescent bulbs (Durotest, Philadelphia) was hung 30 cm above the tank, ensuring females were not dark adapted during the 8 minute acclimation. After acclimation, I recorded the time the female spent associating in the zones adjacent to male stimuli for 8 minutes. After the first trial, the positions of males were switched to control for positional bias. Following reacclimation, the second 8 minute trial was conducted. Two days later, the female and male pair was tested again with the only difference being that I switched the male who received the painted Sc treatment. In the investigation of enhanced Sc phenotypes, the treatment each stimulus male received was also switched across test days (e.g., large Sc treatment on first test day and small Sc treatment on second test day). Previous research in our laboratory has demonstrated that the use of Dr. Naylor’s Blu-Kote dye does not adversely affect male behavior, and that these painted phenotypes elicit female responses similar to natural phenotypes (Hoefler and Morris 1999). I used the total time a female associated with each treatment as an indicator of female mate preference. A female was considered to have a side bias when she did not enter both choice zones across the two trails on that test day.

RT-PCR analyses

Two wild caught males from Cebolla (January 2007) and a wild caught female from Tanute (December 2005) were sacrificed to determine the specificity of proto-
oncogene and oncogene expression. Two tissue samples (approximately 20 mg each) were taken from each individual for purification of total RNA; one sample from the Sc pattern and the other from a nonpigmented region on the side of fish (control). Total RNA from these animal tissues were extracted using Qiagen RNeasy® Mini Kit. The manufacturer’s instructions were followed, including homogenization with QIAshredder™ and an on-column DNase I digestion step (RNase-Free DNase Set, QIAGEN), to extract total RNA. Total RNA was eluted in 50 µl of RNase-Free H2O. Qiagen QuantiTect® Reverse Transcription Kit was used to synthesize first strand cDNA from 26 ng of total RNA. cDNA equivalent to 2.6 ng of total RNA was subjected to amplification (LA Taq™ Kit, TAKARA). The following primers were used to amplify β-actin: sense primer 5´-TGGACTTTGAGCAGGAAATG and antisense primer 5´-AATGCCACATGATTCCATAc (Ling et al. 2006). For the amplification of oncogene and proto-oncogene products, the following primers were developed: sense primer 5´-CTAACCGGACCGTCTTCATG located just upstream of the translation initiation site and the antisense primer 5´-TTGAGGTAGTGATTGTCCAG located at the beginning of exon 2. The final concentration of the primers was 100 nM. cDNA amplification was done under the following conditions: initial denaturation at 95 ºC for 5 min, then 33 cycles of denaturation at 95 ºC for 60 s, annealing at 60 ºC for 60 s, and extension at 72 ºC for 30 s, followed by a final extension at 72 ºC for 5 min. Pilot experiments with the β-actin primer set indicated PCR amplification was exponential between cycles 29 and 35 under these PCR conditions. A 10 µl aliquot of the amplification products were fractionated by electrophoresis on an 8.0% polyacrylamide gel in 1X TBE (45 mM Tris-
borate, 1 mM EDTA) buffer and visualized after staining with SYBR® Green (Invitrogen; diluted to 1X) and UV transillumination. The gel image was taken with a Gel Logic100 system (Kodak).

**RESULTS**

I found that Cebolla females spent significantly more time associating with the large Sc treatment than the small Sc treatment (Figure 3.5; mean large Sc time = 471.6 ± 27.9 s, mean small Sc time = 345.4 ± 26.2 s, t18 = 2.49, P < 0.02). This is important because the Sc pigment pattern increases in size with melanoma formation (Schartl et al. 1995). Theoretical studies suggest that even weak female mating preferences can produce strong selection on male traits (Kirkpatrick and Ryan 1991). Therefore, the results of this first experiment provide compelling evidence for the continued evolutionary maintenance of a known oncogene through its role in the augmentation of a visual signal used in the selection of mates.

When females were given a choice between Sc and non-Sc males, I detected a significant preference for Sc males in two populations of *X. cortezi* (Figure 3.6; Tanute: mean Sc time = 496.8 ± 29.8 s, mean non-Sc time = 289.5 ± 24.3 s, t18 = 4.26, P < 0.001; Cebolla: mean Sc time = 474.9 ± 30.1 s, mean non-Sc time = 327.4 ± 23.6 s, t20 = 2.85, P < 0.01). These two populations are found in separate drainages (Table 3.1) that are genetically divergent (Gutiérrez-Rodriguez et al. 2007); therefore, female preferences for the Sc phenotype appear widespread in *X. cortezi*. Due to the association between Sc and
Xmrk, female preference for Sc pattern males would also act to maintain the Xmrk oncogene. However, there was variation in females’ preferences for Sc males across the three populations sampled (Figure 3.6). Females from Conchita, the population with the highest percentage of Sc females (Table 3.1), discriminated against Sc males, preferring to associate with non-Sc males (Figure 3.6; Conchita: mean Sc time = 343.2 ± 27.4 s, mean non-Sc time = 475.0 ± 28.2 s, t₂₆ = -2.41, P < 0.02). Offspring carrying two copies of Xmrk would have decreased viability (Kallman 1971; Schartl et al. 1998) and the potential for a shorter reproductive lifespan due to the increased risk of melanoma formation (see below). Therefore, the results from the second experiment suggest that when the frequency of Sc is relatively high in females, there is direct selection on females to avoid mating with Sc pattern males in that population.

DISCUSSION

Traditionally, non-hybrid melanoma formation was thought to be an artifact of housing Xiphophorus in the laboratory (Kallman 1971; Schartl et al. 1995) because individuals with melanomas were not found in the field or in museum collections. However, in a single day of collecting X cortezi for this study, I found five males and one female out of the ninety-nine individuals surveyed with melanomas originating from Sc at Conchita. The rate of disease progression in wild populations is not known. In a laboratory setting, the formation and progression of melanomas in Xiphophorus can take months before lethality (Chapter 1), during which time males court and appear able to
mate with females, thereby passing on the \textit{Xmrk} oncogene. However, unlike \textit{X. variatus}, which is only known to develop melanomas during senescence (i.e., > 18 months old; Schartl et al. 1995), the incidence of melanoma formation in non-hybrid \textit{X. cortezi} is highest in sexually active males that are 9 to 12 months old (Schartl et al. 1995). Kallman (1971) documented the presence of severe melanomas in non-hybrid \textit{X. cortezi} by seven months of age. \textit{Xiphophorus} are thought to be able to live two years in natural populations (Reznick and Miles 1989; Schartl et al. 1995), and one study based on otoliths counts of wild caught \textit{Xiphophorus nigrensis} estimated that a 39 mm male (i.e., average male size for individuals in this study) would reach sexual maturity at approximately four and half months of age (Morris and Ryan 1990). Therefore, melanoma formation in \textit{X. cortezi} males occurring within this first year would represent a substantial decrease in an individual’s reproductive lifespan. Whether or not increased male attractiveness associated with the Sc phenotype compensates for the potential reproductive loss due to early mortality is unclear. The greater incidence of melanoma formation in \textit{X. cortezi} males as compared to females (Schartl et al. 1995) is consistent with our results demonstrating a mating advantage for male \textit{X. cortezi} with larger Sc pigment patterns.

It has been hypothesized that female mating preferences for traits that make males more visible could have initially evolved as the result of a pre-existing sensory bias (Endler and Basolo 1998). Once present, these preferences can be maintained through direct selection due to reduced mate search costs (Reynolds and Gross 1990; Westcott 1994). Thus, female preference for Sc patterns (and larger Sc patterns) in males may
reflect a general bias for more visible males (i.e., with more pigmented area). In fact, *X. cortezi* females from the Conchita population have been documented to prefer males with more melanin irrespective of location (i.e., individual bars or a single larger bar with the same area; Morris et al. 2001). However, *X. cortezi* females from Conchita discriminate against Sc patterned males, despite the increased visibility of these males due to their pigmentation. I suggest that that the bias for more pigment in *X. cortezi* males has become more refined, possibly due to the increase in mating costs associated with choosing males with Sc (and *Xmrk*) outweighing the benefits to females of reduced mate search cost. The greater frequency of Sc in females at Conchita would increase the probability of producing offspring with two copies of *Xmrk*. These offspring are more likely to have melanomas that shorten their reproductive lifespan (Kallman 1971; Schartl et al. 1995). In addition, there is evidence that offspring with two copies of *Xmrk* may be nonviable (Schartl et al. 1998). A negative relationship between male ornamentation and an indicator of male viability has also been detected in guppies (Brooks 2000) and such relationships are expected to influence the extent and direction of the selection on female mating preferences.

Explaining how a functional *Xmrk* has been evolutionarily maintained within divergent *Xiphophorus* species has remained a considerable challenge. Remarkably, *Xmrk* has become more efficient since its origin, incorporating two gain of function mutations within its coding region that lead to ligand independent dimerization (Meierjohann and Schartl 2006). Because *Xmrk* is always correlated with specific macromelanophore patterns (Schartl et al. 1995; Weis and Schartl 1998), these patterns
have been suggested to play a key role in maintaining Xmrk (Franck et al. 2001; Meierjohann and Schartl 2006). Despite this, I am aware of only one other study that has detected selection for a macromelanophore pattern (Franck et al. 2001), and this study was conducted on X. helleri, which lacks the Xmrk genotype (Weis and Schartl 1998). It is intriguing that both patterns, dabbed (X. helleri; Kallman and Atz 1966) and spotted caudal (X. cortezi; Kallman 1971), are determined by autosomal genes and are not sex linked. To date, however, the only mechanism that has been proposed to explain the preservation of Xmrk, which is located on the sex chromosomes (Froschauer et al. 2002) is the genetic hitch-hiking model (Maynard Smith and Haigh 1974). Although the genetic hitch-hiking model can increase the frequency of neutral or even slightly deleterious mutations, it necessitates close proximity on a chromosome (Maynard Smith and Haigh 1974). Thus, at least in the case of X. cortezi, which has retained a functional Xmrk oncogene despite the autosomal determination of Sc, this mechanism does not seem applicable.

Rapid morphological evolution, common for traits under sexual selection (Baker and Wilkinson 2001; Ritchie et al. 2007), can increase an individual’s susceptibility to cancer (Graham 1992). I present here a system in which the onset of melanoma formation is advantageous for the acquisition of mates. This is the first evidence of sexual selection acting to favor the evolutionary maintenance of an oncogene. X. cortezi female preference for males with enhanced Sc phenotypes explains the continued genetic correlation between Xmrk and Sc and may have an epistatic basis (Takahasi and Tajima 2005) with the mating advantage associated with larger Sc compensating for the
deleterious Xmrk. The fact that all Xiphophorus species that have maintained a functional Xmrk have a coupled macromelanophore pigment pattern (Weis and Schartl 1998) provides additional support that these genetic entities work synergistically in their capacity to enhance visual signals used in sexual selection.
LITERATURE CITED


Table 3.1 Percentage of Sc phenotype by sex across three study sites. All adult individuals collected were scored upon capture in December 2005 (Tanute, Conchita) and January 2007 (Cebolla). Juveniles were excluded from survey because of variance in the developmental timing of Sc expression.

<table>
<thead>
<tr>
<th>Population</th>
<th>Drainage</th>
<th>Sex</th>
<th>Sc individuals</th>
<th>Total individuals</th>
<th>Sc %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tanute</td>
<td>Tampaón</td>
<td>Male</td>
<td>19</td>
<td>63</td>
<td>30.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>1</td>
<td>47</td>
<td>2.1</td>
</tr>
<tr>
<td>Cebolla</td>
<td>Moctezuma</td>
<td>Male</td>
<td>31</td>
<td>75</td>
<td>41.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>0</td>
<td>30</td>
<td>0.0</td>
</tr>
<tr>
<td>Conchita</td>
<td>Moctezuma</td>
<td>Male</td>
<td>23</td>
<td>81</td>
<td>28.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>9</td>
<td>57</td>
<td>15.8</td>
</tr>
</tbody>
</table>
Figure 3.1 Xmrk and proto-oncogene expression in Xiphophorus cortesi. Semiquantitative RT-PCR analyses using the same template primers for the oncogene (Xmrk, bottom bands) and proto-oncogene (top bands). In each of the three wild caught individuals, all of which did not have visible melanoma formation, the Sc tissue was associated with both Xmrk and proto-oncogene expression, whereas in the nonpigmented control (C) tissue only the proto-oncogene was expressed. The housekeeping gene β-actin is included as a loading control (Ling et al. 2006). A 15 bp insertion associated with the proto-oncogene in X. cortesi accounts for the fractionation of the two bands by gel electrophoresis (Fernandez and Tanda unpublished data). This small insertion was found in the predicted signal peptide sequence at the beginning of the proto-oncogene. Polymorphisms within this region of the oncogene and proto-oncogene are common in individuals derived from wild populations of several Xiphophorus species (Schartl et al. 1998).
Figure 3.2 Sc phenotype in Xiphophorus cortezi. (A) X. cortezi male collected from Tanute with an average expression of Sc on caudal fin (non-malignant). Scale bar: 5 mm. (B) X. cortezi male from Conchita with malignant melanoma extending from Sc into the caudal peduncle. Histopathology confirms malignant melanoma in this individual classified as a ‘melanophorous-macromelanophorous polymorphic melanoma’ (Gimenez-Conti et al. 2001; Fernandez and Bowser unpublished data). Note on this male that a substantial portion of the sword and caudal fin has sloughed off, which ultimately impairs swimming ability. Specimens were photographed on the day they were collected in December 2005. Scale bar: 5 mm.
Figure 3.3  Experiment 1 treatments. (A) *Xiphophorus cortezi* male painted with Dr. Naylor’s Blu-Kote dye to represent the small Sc treatment used in experiment 1. Small Sc treatment area was calculated to approximate the 20th percentile of Sc phenotypic expression for wild caught males at Cebolla. Scale bar: 5 mm. (B) The same *X. cortezi* male with a representative painting of the large Sc treatment used in experiment 1 which was calculated to approximate the 80th percentile Sc phenotypic expression for wild caught males at Cebolla. Scale bar: 5 mm.
Figure 3.4  Experiment 2 treatment. *X. cortezi* male with a representative example of the average size Sc treatment used in experiment 2. The average size treatment was determined for each of the 3 populations tested in experiment 2 and was calculated to approximate the 50th percentile Sc expression of wild caught males from each location. During both experiment 1 and 2 care was taken to minimize variations in perimeter length and total number of contiguous areas. Scale bar: 5 mm.
Figure 3.5 Female preferences for enhanced Sc phenotypes. Time females from the Cebolla population spent associating with large Sc treatment as compared to small Sc treatment, as well as with an average size Sc as compared to no Sc. Bars represent the mean ± SEM.
Figure 3.6 Female preferences for Sc across populations. Primary Y axis (left, grey bars) represents the mean amount of time females spent with painted Sc male minus the time spent with the non-Sc males across all 4 trials. Positive values indicate preference for Sc males whereas negative values indicate discrimination against Sc males. Secondary Y axis (right, white bars) represents the proportion of females with Sc phenotype for each of the three populations (all Cebolla females lacked Sc). Bars represent the mean ± SEM.
CHAPTER 4

FREQUENCY DEPENDENT SELECTION AND THE XMRK ONCOGENE IN XIPHOPHORUS CORTEZI: BALANCING OFFSPRING ATTRACTIVENESS AND OFFSPRING SURVIVAL
ABSTRACT

Cancer genes, especially those transmitted via gametes, represent an evolutionary paradox. In the Xiphophorus melanoma model, a functional germline oncogene (Xmrk) has been maintained for millions of years despite being deleterious. In non-hybrid Xiphophorus cortezi, malignant melanomas originate from spotted caudal (Sc) pattern in the caudal fin. X. cortezi with the Sc phenotype have the Xmrk genotype; however, due to the incomplete penetrance of Sc, individuals without the Sc pattern can also have the Xmrk oncogene. Previous research demonstrated that X. cortezi females from two populations, in which the frequency of Sc is male biased, have mate preferences for the Sc pattern in males. In a third population that had higher frequencies of Sc in females, females discriminated against Sc males, preferring non-Sc males. Here, I examine female preferences for the Sc trait in three additional populations of X. cortezi and estimate the frequency of Sc as well as Xmrk across all six natural populations of X. cortezi. In the three populations tested in the current study, no preference was detected in two populations, however in the third population, preferences for Sc were found despite the lack of Sc or Xmrk in this population. The ratio of males to females with Xmrk across populations was either male biased or approximately equal and the frequency of the Xmrk oncogene ranged from 0% in one population to almost 70% in two populations. The relationship between strength of female preference for Sc and Xmrk frequencies across the six populations suggests that Xmrk is under frequency dependent selection due to the decreased fitness of homozygous Xmrk offspring.
Evolutionary trade-offs frequently occur in sexual selection and more recently have become a central theme underlying the evolutionary biology of cancer (Graham 1992; Greaves 2000; Crespi and Summers 2006). Although many cancers occur from somatic mutation or environmental risk factors, some genes capable of producing tumors are transmitted through the germ line. Therefore, despite their deleterious nature, such oncogenes can be maintained over evolutionary time periods across many generations (Meierjohann and Schartl 2006). A well known example is the X-linked androgen receptor (\textit{AR}) gene that binds testosterone (Ross et al. 1998), a hormone that can increase not only reproductive fitness in early adulthood but also the risk of developing prostate cancer later in life (Summers and Crespi 2008). Seemingly, the potential for increased reproductive benefits in young males due to effects of testosterone outweighs its deleterious effects late in life when reproductive fitness is decreased (Williams 1957). This highlights an important concept regarding the continued evolutionary maintenance of oncogenes; the relative costs associated with cancer depend on several factors including when the disease manifests itself, rate of progression, and the affected area (Graham 1992).

In the \textit{Xiphophorus} melanoma model, a germline oncogene (\textit{Xiphophorus} melanoma receptor kinase, \textit{Xmrk}) that originated from a tandem gene duplication of the \textit{Xiphophorus} epidermal growth factor receptor (\textit{Egfr}) gene has remained functional despite being deleterious (Schartl et al. 1995; Weis and Schartl 1998) and located in an
extremely unstable genomic region (Volff et al. 2003). The proto-oncogene *Xiphophorus Egfr* is the fish ortholog to mammalian *Egfr* (for review Meierjohann and Schartl 2006). The event that led to the creation of *Xmrk* has been suggested to predate the divergence of *Xiphophorus* fishes with *Xmrk* being subsequently lost several times (Kazianis and Borowsky 1995; Weis and Schartl 1998). *Xmrk* is not only sufficient to induce melanomas within *Xiphophorus*, but 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, spontaneous non-hybrid melanomas have occurred under laboratory conditions in 3 of the 9 species that have retained *Xmrk* (Kallman 1971; Borowsky 1973; Chapter 1). In both hybrid and non-hybrid melanomas, the overexpression of *Xmrk* oncogene leads to melanomas originating from melanophores of specific macromelanophore patterns (Schartl et al. 1995, Weis and Schartl 1998). Such malignancies are costly because of progression into underlying muscle tissue, which can ultimately impair swimming ability (for non-hybrid tumor histology see Schartl et al. 1995; Chapter 1). Thus, explaining the evolutionary conservation of this germline oncogene has remained a challenge for evolutionary biologists. Because macromelanophore patterns (M patterns) are always correlated with *Xmrk* (Schartl et al. 1995; Weis and Schartl 1998), recent work has suggested these patterns may play a key role in the evolutionary maintenance of *Xmrk* (Franck et al. 2001; Meierjohann and Schartl 2006; Chapter 3).

Non-hybrid melanomas were initially believed to be an artifact of housing *Xiphophorus* in laboratories (Kallman 1971; Borowsky 1973). However, a recent study
has observed melanomas in wild caught *Xiphophorus*, which indicates there are costs associated with carrying *Xmrk* in a natural setting (see Chapter 3). Under a laboratory setting, there are differences in individual susceptibility to melanoma formation and the incidence of non-hybrid malignancies across species (Kazianis and Borowsky 1995; Schartl et al. 1995). For example, melanoma formation within *X. variatus* is approximately equal between sexes and is only known to occur during senescence (i.e., >18 months; Kazianis and Borowsky 1995; Schartl et al. 1995). However, within *X. cortezi*, melanomas are most common within the first year of life, with males being more susceptible than females (Schartl et al. 1995). Moreover, Schartl and colleagues (1995) suggest that malignancies within *X. cortezi* are most frequent in sexually active males of ‘high social rank’. This is important because undoubtedly the costs associated with spontaneous melanoma formation depend on when they occur during an individual’s lifetime (e.g., pre-reproduction or post-reproduction).

Because *X. cortezi* forms melanomas during the reproductive period, when costs associated with malignancies are increased, this species is ideal for investigating the possible benefits the *Xmrk* oncogene confers to the individual carrier. *X. cortezi* is polymorphic for *Xmrk* and the associated M pattern spotted caudal (Sc; Kallman 1971; Schartl et al. 1995). 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. Pedigree crossing experiments suggest that Sc is under autosomal determination (Kallman 1971) although it has not been characterized at the genomic or DNA molecular level. *Xmrk* is an essential component for
the expression of the Sc phenotype in *X. cortezi* (Chapter 3; Schartl et al. 1995; Weis and Schartl 1998). Because *Xmrk* is located on the sex chromosomes (X and/or Y chromosomes; Froschauer et al. 2002), it is passed on to successive generations through the germ line and malignant melanomas originating from Sc M pattern occur in both sexes of *X. cortezi* (Kallman 1971; Schartl et al. 1995). Thus, all individuals with the Sc phenotype have the *Xmrk* locus. However, because of the incomplete penetrance of Sc, individuals that lack phenotypic expression of Sc can also have one or two copies of *Xmrk* (Kallman 1971).

Previously, in Chapter 3 it was demonstrated that female *X. cortezi* prefer spotted caudal (Sc) males to males without the Sc phenotype in two populations (Tanute and Cebolla) in which the frequency of Sc phenotype was male biased (for Sc frequency data: Table 4.1; for preference data: Table 4.2). This study also found that females preferred males with enhanced Sc patterns to size-matched males with reduced Sc patterns. However, there was variation in female preference for the Sc phenotype. Females from a third population, Conchita, which had the highest percentage of Sc females (Table 4.1), discriminated against Sc males, preferring to associate with non-Sc males (Chapter 3; Table 4.2). Because all individuals with Sc have *Xmrk* (Schartl et al. 1995; Weis and Schartl 1998), the observed variation in female preference was attributed to decreased indirect benefits associated with the mating of Sc males and Sc females. Offspring carrying two copies of *Xmrk* have reduced viability (Kallman 1971; Schartl et al. 1998) and the potential for a substantially decreased reproductive lifespan (see Chapter 3). However, *X. cortezi* individuals were not genotyped for the *Xmrk* oncogene in the
previous study. If offspring carrying two copies of $Xmrk$ have less fitness, the mating decisions $X. cortezi$ females should be influenced by the frequency of $Xmrk$ oncogene in males and females within that population. Because the Sc M pattern associated with $Xmrk$ in this species has incomplete penetrance, determining the $Xmrk$ genotype of individuals who do not express the Sc phenotype within a population is important in supporting the previously proposed hypothesis in Chapter 3. In addition, despite the considerable scientific attention devoted to the $Xiphophorus$ melanoma model since its inception in the 1920s, no study has reported on the frequency of $Xmrk$ in the wild from any $Xiphophorus$ species.

This study had three objectives. First, I wanted to examine the extent of female preferences for the Sc phenotype in $X. cortezi$ males across their geographic distribution. Therefore, three additional populations (Chalpuhuacanita, San Martín, and Tecolutlo) of $X. cortezi$ were collected from the Tempoal river drainage (Río Pánuco basin), which were not sampled in Chapter 3. The second objective was to estimate the frequencies of the Sc phenotype and the $Xmrk$ genotype across the six sampled populations of $X. cortezi$. Finally, the last objective was to compare mate preferences for Sc with the frequencies of Sc and the $Xmrk$ oncogene within each population to assess if the maintenance of this oncogene within $X. cortezi$ could represent an evolutionary trade-off between offspring attractiveness and offspring survival.
METHODS

Because I was interested in making comparisons between the preference data collected in this Chapter and that of Chapter 3, all methodology including but not limited to animal husbandry, the pairing of male stimuli, application of male treatments, and female preferences tests was identical to that used in Chapter 3.

**Specimen collection and housing**

All individuals were collected from the following six natural populations and represent all three drainages within the natural distribution of *X. cortezi* (Rauchenberger et al. 1990): Arroyo Tanute (Tampaón drainage) N 21 39 123, W 99 02 127; Arroyo Cebolla (Moctezuma drainage) N 21 23 472, W 98 59 885; Arroyo Conchita (Moctezuma drainage) N 21 33 500, W 98 59 320; Arroyo Chalpuhuacanita (Tempoal drainage) N 21 12 364, W 98 40 153; Río San Martín (Tempoal drainage) N 21 22 173, W 98 39 543; and Arroyo Tecolutlo (Tempoal drainage) N 21 07 270, W 98 28 075 (Hidalgo & San Luis Potosí provinces; Mexico). The following *Xiphophorus* were sympatric with *X. cortezi* at the six collection sites: *X. variatus* (Arroyo Tanute, Arroyo Chalpuhuacanita, Río San Martín and Arroyo Tecolutlo), *X. multilineatus* (Arroyo Tanute), *X. birchmanni* (Arroyo Chalpuhuacanita and Río San Martín). *X. cortezi* was the only *Xiphophorus* observed at Arroyo Conchita and Arroyo Cebolla. With the exception of Cebolla, collection sites were selected to maximize sampling within the known distribution of *X. cortezi* (Rauchenberger et al. 1990). Site selection was also based upon the phylogenetic reconstruction of *X. cortezi* haplotypes (Gutierrez-Rodriguez et al. 2007). Cebolla a site
not described in Rauchenberger et al. (1990), but is located upstream from Conchita, and was sampled after substantial habitat degradation occurred at Conchita in late 2006. Females and stimulus males used in dichotomous choice tests were collected as adults during three field seasons: December 2005 (Conchita and Tanute), April 2006 (Chalpuhuacanita, Tecolutlo, San Martin) and January 2007 (Cebolla). Upon return to the United States, all females used in preference tests were initially housed in communal tanks with males from the same locale. A minimum of 4 weeks prior to conducting female preferences tests females were individually housed in 19 L tanks and visually isolated from one another. Females were measured for standard length (defined as tip of the snout to end of the caudal peduncle) and scored for the presence/absence of Sc before being placed in their isolation tanks. All fish were maintained under standard laboratory conditions throughout the experiments which included a 12L:12D cycle, daily feeding (Tetramin® flakes), and a constant temperature of 22°C.

**Male treatments**

Pairs of males were matched for standard length within ± 2 mm. Because *X. cortezi* females prefer males with symmetrical barring (Morris 1998; Morris and Casey 1998) and have polymorphic preferences for males with vertical bars (Morris et al. 2003), males were also paired in accordance with their bar state, bar symmetry and overall similarity in melanin pigment patterning. Paired males did not differ by more than 3 total vertical bars (both sides) and were always from the same population as the focal female. All stimulus males lacked the Sc phenotype and male pairs were randomly assigned to
each female. Seven pairs of stimuli males were used for choice tests on San Martín and Tecolutlo females, and six pairs of males were used for tests conducted on Chalpuhuacanita females. In order to investigate female preferences for the Sc phenotype, one male in each pair was randomly chosen to receive a painted Sc treatment of average size using Dr. Naylor’s Blue-Kote dye (H. W. Naylor Co., Inc., Morris, NY; Hoefler and Morris 1999) while the other male received a mock (water) treatment. The size of the Sc phenotype applied to males was consistent and was representative of the average Sc expression for the population being tested based on measurement of wild caught males from that site. In the case of Tecolutlo, in which all individuals surveyed and collected lacked Sc (Table 4.1), the Sc phenotype was painted based upon measurements of Sc at the other five *X. cortezi* populations. Care was taken to ensure that handling time (~ 40 seconds) was consistent between treatments (painting vs. mock painting) and across individuals. All observation trials, including the painting of male treatments, were conducted by one person (A.A.F.) to reduce variation in the painting of males across females.

**Female preference tests**

Female preference tests were conducted on twenty-five females from Chalpuhuacanita, twenty-seven females from San Martín, and twenty-two females from Tecolutlo. I used a standard dichotomous choice test design (for tank description see Morris et al. 2001). A twin light fixture with Vitalite® fluorescent bulbs (Durotest, Philadelphia) was hung 30 cm above the tank, ensuring females were not dark adapted
during the 8 minute acclimation. After acclimation, I recorded the time the female spent associating in the zones adjacent to male stimuli for 8 minutes. After the first trial, the positions of males were switched to control for positional bias. Following reacclimation, the second 8 minute trial was conducted. Two days later, the female and same male pair was tested again with the only difference being the treatment each male received was switched across test days. Switching males between treatments controlled for any unforeseen behavioral or phenotypic differences between the males in a pair that females might have mate preferences for. Within 3-4 hours the antiseptic dye fades which makes it possible to switch the male that received the painted Sc treatment across test days. Previous research in our laboratory has demonstrated that the use of Dr. Naylor’s Blu-Kote dye does not adversely affect male behavior, and that these painted phenotypes elicit female responses similar to natural phenotypes (Hoefler and Morris 1999). I used the average time a female associated with each treatment across test days as an indicator of female mate preference. Association time has been demonstrated to be a reliable indicator of female mating preferences in viviparous fishes because it is positively correlated with male mating success in the field (X. nigrensis; Ryan et al. 1990; Morris et al. 1992) and the laboratory (Gambusia holbrooki; Bizsarra 2001). In addition, a recent study conducted on X. nigrensis found that association time is a more consistent and repeatable estimate of female preference than other female behaviors (including receptivity behaviors; Cummings and Monhagen 2006). A female was considered to have a side bias when she did not enter both choice zones across the two trials on that test day.
A single female from Chalpuhuacanita demonstrated a side bias and was not included in
the statistical analysis.

**Field data and tissue collection**

Additional males and females were randomly sampled from each of the six sites
in order to estimate the frequencies of the Sc phenotype and Xmrk genotype across the
geographic distribution of *X. cortezi* (Table 4.1). Individuals were collected using three
different techniques: electroshock, seine, and bait traps. All individuals that were not
collected for behavioral assays were digitally photographed on site and a small piece of
the caudal fin was removed and preserved in 95% ethanol for subsequent DNA
extraction. After the collection trips, digital photographs of individual fish were scored
for the presence/absence of the Sc phenotype.

**DNA analysis**

DNA was extracted from fish tissue (caudal fin clips) using DNeasy® tissue kit
(Qiagen Inc.) following the manufacturer’s instructions. Total elution volume was 100 µl.
The presence of an oncogenic copy of the *EGFR* was determined by cross-referencing the
polymerase chain reaction (PCR) products of two primers sets developed in the
laboratory. 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-B). The following primer set was used to screen for the presence of the \textit{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’- ACAAGTTTGTGGAAAATAACCTGAACCTC 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 \textit{Xmrk} oncogene (\textit{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’- TCAGTTTGGATCAGAGATG 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) yields a total of three bands: proto-oncogene band at ~750 bp and two oncogene bands, one at ~950 bp and one at ~1100 bp. 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).

**Statistical analysis**

Paired sample t-tests were conducted for each population to determine if there was a significant difference in the amount of time females associated with the Sc male and the non-Sc male. A single factor analysis of variance was conducted to determine if there was a significance difference in female strength preference for Sc males across the six populations. Strength of preference in females was calculated as the average amount of time a female associated with the Sc male stimulus minus the average time that female associated with the non-Sc male stimulus. The data in all three populations were normally distributed and the condition of homogeneity of variance was met in the analysis of variance.
RESULTS

The frequency of the Sc phenotype across the six populations surveyed varied from completely lacking in one population (Tecolutlo) to approximately thirty-five percent in the Cebolla population (Table 4.1). Variation in the frequencies of Sc between the sexes was even more striking; with populations having either relatively equal ratios of Sc between males and females or being male biased (females biased not observed). The frequency of Sc in *X. cortezi* from Conchita and Chalpuhuacanita had relatively equal ratios between males and females whereas the occurrence of Sc was male biased at the Tanute and Cebolla collection sites (Table 4.1). The frequency of Sc at San Martín was very low with only one male and one female having Sc (Table 4.1).

Of the 552 *X. cortezi* collected, 187 individuals had the *Xmrk* oncogene. All 99 individuals with the Sc phenotype also had the *Xmrk* genotype, confirming that the *Xmrk* oncogene in *X. cortezi* is required for the phenotypic expression of the Sc M pattern. The frequency of the *Xmrk* oncogene was greater than that of the Sc phenotype in all populations with the exception of Tecolutlo (Table 4.1). *Xmrk* was not detected at Tecolutlo. The differences in the frequency of *Xmrk* and Sc are the result of the incomplete penetrance of Sc (i.e., individuals that lack Sc phenotype but have *Xmrk* genotype). Across populations, the frequency of the *Xmrk* oncogene ranged from zero to nearly seventy percent. As with Sc, the populations had either relatively equal frequencies of *Xmrk* across males and females or were male biased (Table 4.1). The differential association of *Xmrk* across sexes was most dramatic at Cebolla, where almost
eighty-seven percent of males had \textit{Xmrk} yet only fourteen percent of females had \textit{Xmrk} (Table 4.1).

Tecolutlo females spent significantly more time associating with the Sc treatment than the non-Sc treatment (Table 4.2) despite the absence of the Sc phenotype in the individuals surveyed from this population. However, females from San Martín and Chalpuhuacanita did not exhibit a significant preference for either treatment in males (Table 4.2; Figure 4.1). In addition, there was a significant effect of population on the female strength of preference for the Sc phenotype in males (one way ANOVA: $F_{5, 135} = 6.3$, $P < 0.0001$).

**DISCUSSION**

Female preferences for Sc patterned males, and therefore \textit{Xmrk} males, are widespread within the known geographic distribution of \textit{Xiphophorus cortezi}. Preferences for the Sc phenotype in males were detected in all three drainages in which this species is found (Tempoal: this Chapter; Moctezuma and Tampaón: Chapter 3). Mate preference for Sc males at Tecolutlo despite the lack of this phenotype in the 85 individuals sampled indicates preferences for Sc could represent a bias for pigmentation (see discussion in Chapter 3). Previous research in \textit{X. cortezi} supports this hypothesis as females were found to prefer more melanin pigmentation to less (Morris 1998; Chapter 3). In addition, females do not discriminate between patterns that are spaced apart and those that are clumped together when total pigment is held constant (Morris et al. 2001). Because
*Xiphophorus* fishes are a non-resource based mating system (Farr 1989), *X. cortezi* females do not receive direct benefits associated with resource allocation or paternal care, but females who exhibit preferences for Sc/\(Xmrk\) could decrease the costs of finding a mate due to the presence of Sc in males (Reynolds and Gross 1990; Jennions and Petrie 1997). In populations with preferences for Sc males, females preferring Sc males would also receive indirect (genetic) benefits as well because such preferences would increase the probability of females producing attractive sons (‘sexy’ sons; Weatherhead and Robertson 1979). There are also benefits to *X. cortezi* males carrying the Sc and/or \(Xmrk\) despite the increased risk of melanoma formation in this sex. Not only do *X. cortezi* males with Sc and \(Xmrk\) receive mating advantages associated with mate choice, but they gain advantages through male-male competition as well (see Chapter 2). *X. cortezi* males with Sc/\(Xmrk\) and males with only \(Xmrk\) (due to incomplete penetrance of Sc) from these same populations are more aggressive in mirror image trials than males without the \(Xmrk\) genotype (see Chapter 2). Collectively, these results (Chapters 2, 3, and 4) demonstrate that there can be benefits to both sexes of carrying Sc and/or \(Xmrk\), however the magnitude of these benefits depends on the relative frequencies of \(Xmrk\) in males and females within that population (see below, Chapter 3).

The frequency of \(Xmrk\) in several populations of *X. cortezi* is remarkably high compared to other hereditary genes involved in oncogenesis. For example a mutation of *BRCA1*, a hereditary tumor suppressor gene, leads to an increased risk of developing breast and ovarian cancer and such mutations are found in populations in general at a frequency of \(\sim 0.001\%\) (1 in 833; Ford et al. 1995) and in populations with an elevated
risk of cancer at ~2% (due to founder effects; Szabo and King 1997). By comparison, this study revealed that $Xmrk$ is found at ~34% in $X. cortezi$ in general (mean of all 6 populations surveyed; 187 of 552 individuals sampled) and approaches a frequency of 70% in two populations sampled (Cebolla and Conchita). Because $Xmrk$ in $X. cortezi$ increases not only the risk of mortality in parents (melanomas) but also in offspring through germline transmission, such frequencies must be countered by a fitness advantage associated with the $Xmrk$ genotype. However, such benefits appear insufficient to maintain $Xmrk$ at high frequencies in both sexes because of reduced benefits under such circumstances (see Chapter 3).

The association of $Xmrk$ with each sex across populations (Table 4.1) supports the previous explanation for the observed variation in female preferences for Sc males in Chapter 3 that was based only on Sc frequency in populations. Female preference for Sc/$Xmrk$ males at least in part depends on the relative frequencies of the $Xmrk$ oncogene within males and females of that population. Therefore, $Xmrk$ is under frequency dependent selection. The frequency of $Xmrk$ and female preference data collected from two sites (Conchita and Cebolla), which are relatively close in proximity (roughly 1 km), supports this hypothesis. Conchita females discriminate against Sc males and prefer to associate with non-Sc males whereas Cebolla females prefer Sc males to non-Sc males (Table 4.2; Figure 4.1). The frequency of $Xmrk$ is approximately equal at the two sites (Conchita: 66.2%; Cebolla: 69.8%), however $Xmrk$ is much more common in females from Conchita than Cebolla (54.5% and 13.6%, respectively). This would result in an increased likelihood of producing offspring with two copies of $Xmrk$ at Conchita (i.e.,
*Xmrk* male and *Xmrk* female mating). Therefore, the female preference data not only supports evidence from previous studies, which indicated that two copies of *Xmrk* within an individual is detrimental (Kallman 1971; Schartl et al. 1998), but also that *Xmrk* is under frequency dependent selection due in part to decreased offspring fitness.

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-minded approach has led to our understanding that the maintenance of genes with oncogenetic potential can result from genomic conflict (‘selfish’ genes: Summers et al. 2002; Kleene 2005), antagonistic coevolution (maternal-fetal interactions: Lala et al. 2002; Summers and Crespi 2005), antagonistic pleiotropy (Summers and Crespi 2008), and sexual selection (Chapter 2 and 3). For example, a recent study hypothesized that the evolution of viviparity (live bearing young as in *Xiphophorus*) results in an increased vulnerability to cancer (Hayakawa 2006). Hayakawa (2006) suggests that decreases in the adaptive maternal immune response, thereby allowing her body to not reject developing embryo(s), concomitantly increases the risk of normal cellular processes becoming aberrant due to the failure of the diminished immune system in recognizing such abnormalities (termed ‘tumor escape’). Although *Xiphophorus* are livebearing fish, it is not clear whether such a mechanism underlies the overexpression of the *Xmrk* oncogene within the *Xiphophorus* melanoma model. Recent work on the well-known tumor suppression gene *TP53* has provided another example of antagonistic pleiotropy analogous to the trade-off between testosterone and susceptibility to prostate
cancer (see introduction). We now know that $TP53$ is not only effective at suppressing cancer, but also stem cells that replenish worn out tissues (for review Rodier et al. 2007). Thus, $TP53$ decreases tumor susceptibility while accelerating senescence (Tyner et al. 2002; Weinstein and Ciszek 2002; Campisi 2005; Krtolica 2005).

The occurrence of melanomas early in the lifespan of males (~7-12 months; Kallman 1971; Schartl et al. 1995) and later in the lifespan of females is consistent with the 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 other 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 and Miles 1989) and therefore 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 and male-male competition (Chapter 2), can therefore offset the occurrence of malignancies earlier in males (so long
as sexual maturity is reached). This is particularly true if the enhancement of Sc due to the \textit{Xmrk} oncogene (i.e., melanosis/melanomas) is beneficial in male competition or mate choice decisions (Chapter 3). The prevalence of non-hybrid melanomas across species also conforms to these principles. Under laboratory conditions, melanomas occur in approximately 42\% of \textit{X. variatus} but only occur after 18 months of age (Kazianis and Borowsky 1995) when selection against senescence is relaxed. However, the frequency of melanomas in \textit{X. cortezi} in the laboratory is less than 10 \% (Kallman 1971; Schartl 1995) due, at least in part, to their occurrence earlier in adulthood (Williams 1957).

Fluctuations within the physical and/or social environment are critical to the origin of evolutionary trade-offs and can serve as the basis for balanced polymorphism among phenotypes (Andersson 1994; Sinervo and Calsbeek 2006). Within \textit{Xiphophorus}, the prevalence of diverse genetic melanin polymorphisms is an enigma to evolutionary biologists and has led investigators to search for potential mechanisms capable of maintaining such phenotypic variation. Borowsky (1978; 1981; 1984) found that the tailspot morphs (crescent, cut-crescent) of \textit{X. variatus} differ in their relative mean sizes and body conditions in the field. Such differences were attributed to variations in their metabolic rate and he suggested that these two morphs are specialists adapted to resource availability within the environment (Borowsky 1978). However, the function of melanin patterns as sexually selected signals within \textit{Xiphophorus} can also contribute to the maintenance of balanced polymorphisms. For example, \textit{X. helleri} females prefer males with dabbed (Db) melanin pattern over non-Db males under turbid but not clear water conditions, and therefore, the polymorphism may be maintained in a seasonally variable
environment (Franck et al. 2001). Contrary to \textit{X. variatus}, a recent study found there was no difference in the metabolic rates of \textit{X. helleri} with and without Db patterns (Meyer et al. 2006). Within \textit{X. cortezi}, female preferences for vertical body bars in males depend on the presence of this trait in females, and this polymorphic mate preference likely plays a role in the persistence of barred and barless male morphs (Morris et al. 2003). The findings of the current and previous study (Chapter 3) within \textit{X. cortezi} indicate that variation in female preferences is contingent on the relative frequencies of the Sc phenotype and \textit{Xmrk} oncogene in males and females within a population (frequency dependent selection). Frequency dependent selection has been demonstrated to maintain polymorphic traits under numerous circumstances and in many taxa (for review Sinervo and Calsbeek 2006), however to my knowledge, this is the first example of frequency dependent selection maintaining a melanin polymorphism within \textit{Xiphophorus}.

The lack of female preferences for either phenotype (Sc or no Sc) in males from Chalpuhuacanita and San Martín is intriguing. The lack of preference at Chalpuhuacanita is most surprising because although the frequencies of \textit{Xmrk} were not as high at Conchita, the occurrence of \textit{Xmrk} was approximately equal between the sexes (Table 4.1). Thus, given the costs described above, females in this population might increase their reproductive fitness by not mating with \textit{Xmrk} and Sc males (i.e., discriminate against Sc males). There are a couple of reasons that could attribute to the lack of preferences at these two localities. First, both populations are located within the San Pedro River, which is a tributary of the larger Tempoal River. Therefore, the lack of preference could be the result of founder effects and genetic drift. If drift has influenced
female preferences for Sc at these two sites, then the lack of preference for Sc should also be found in the parental population as well. A recent phylogeographic study found that *X. cortezi* from these two sites are more genetically similar to one another than they are to any of the other populations sampled (Gutierrez-Rodriguez et al. 2007). If the *X. cortezi* females, who colonized this river, did not possess mate preferences for Sc (and there is a genetic component to such preference) then the lack of female preference might be expected. Second, Chalpuhuacanita and San Martín were the only sites where *X. birchmanni* was found during the sampling for this study. *X. birchmanni* is one of the nine *Xiphophorus* species that has retained *Xmrk* and it is also polymorphic for the Sc phenotype (Rauchenberger et al. 1990). Therefore, *X. cortezi* females might not use this trait in mate choice decisions due to the increased probability of hybridizing with *X. birchmanni* males. Additional sampling from allopatric populations of *X. cortezi* along the San Pedro River as well as sampling within the Tempoal River could elucidate whether the two possibilities discussed here underlie the lack of preference in females from Chalpuhuacanita and San Martín.

The results of this study indicate that the persistence of *Xmrk* oncogene within *Xiphophorus cortezi*, like other evolutionarily stable oncogenes, is the result of trade-off between increased offspring attractiveness and decreased offspring survival. Female preference data across populations suggests that the amount of indirect benefits a female receives from mating with *Xmrk/Sc* males depends on frequency of *Xmrk* females within the population and her own *Xmrk* genotype. Thus, the ability of sexual selection to mitigate the deleterious effects (increased risk of cancer, decreased offspring fitness) of
Xmrk is frequency dependent. Because homozygous Xmrk individuals have decreased fitness but the presence of the Xmrk genotype and Sc phenotype within an individual results in mating advantages (male-male competition: Chapter 2; female mate choice: Chapters 3, 4), the theory of heterozygous advantage (Fisher 1922; 1930) also appears to be an important factor in the evolutionary maintenance of the Xmrk oncogene within Xiphophorus.
LITERATURE CITED


Fernandez A. A. and M. R. Morris. in press. Mate choice for more melanin as a mechanism to maintain a functional oncogene. The Proceedings of the National Academy of Sciences of the United States of America.


Table 4.1 Frequency of $Xmrk$ and Sc across populations. The frequencies of the Sc phenotype and the $Xmrk$ genotype across the six *X. cortezi* populations surveyed in males, females and overall (both sexes). Parentheses indicate the actual number of individuals. In the last two columns I estimate the penetrance of Sc phenotype and summarize female preferences for Sc in males from each population. The penetrance of Sc was calculated as the number of Sc individuals divided by the number of $Xmrk$ individuals in any given row. For each population, the number of individuals (N) includes those surveyed in the field and released as well as those collected for behavioral studies.

<table>
<thead>
<tr>
<th>Population</th>
<th>Sex</th>
<th>N</th>
<th>Sc %</th>
<th>$Xmrk$ %</th>
<th>Penetrance of Sc (%)</th>
<th>Preference for Sc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tanute</td>
<td>Male</td>
<td>52</td>
<td>28.8 (15)</td>
<td>55.8 (29)</td>
<td>51.7</td>
<td>Prefer Sc</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>41</td>
<td>2.4 (1)</td>
<td>14.6 (6)</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall</td>
<td>93</td>
<td>17.2 (16)</td>
<td>37.6 (35)</td>
<td>45.6</td>
<td></td>
</tr>
<tr>
<td>Cebolla</td>
<td>Male</td>
<td>74</td>
<td>44.6 (33)</td>
<td>86.5 (64)</td>
<td>51.6</td>
<td>Prefer Sc</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>22</td>
<td>0 (0)</td>
<td>13.6 (3)</td>
<td>0.0</td>
<td>Prefer Sc</td>
</tr>
<tr>
<td></td>
<td>Overall</td>
<td>96</td>
<td>35.4 (33)</td>
<td>69.8 (67)</td>
<td>50.7</td>
<td></td>
</tr>
<tr>
<td>Conchita</td>
<td>Male</td>
<td>38</td>
<td>31.6 (12)</td>
<td>76.3 (29)</td>
<td>41.4</td>
<td>Prefer non-Sc</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>33</td>
<td>24.2 (8)</td>
<td>54.5 (18)</td>
<td>44.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall</td>
<td>71</td>
<td>28.2 (20)</td>
<td>66.2 (47)</td>
<td>42.6</td>
<td></td>
</tr>
<tr>
<td>Chalpu.</td>
<td>Male</td>
<td>45</td>
<td>33.3 (15)</td>
<td>37.8 (17)</td>
<td>88.2</td>
<td>No preference</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>46</td>
<td>26.1 (12)</td>
<td>34.8 (16)</td>
<td>75.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall</td>
<td>91</td>
<td>29.7 (27)</td>
<td>36.3 (33)</td>
<td>81.8</td>
<td></td>
</tr>
<tr>
<td>San Martin</td>
<td>Male</td>
<td>63</td>
<td>1.6 (1)</td>
<td>4.8 (3)</td>
<td>33.3</td>
<td>No preference</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>53</td>
<td>1.9 (1)</td>
<td>3.8 (2)</td>
<td>50.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall</td>
<td>116</td>
<td>1.7 (2)</td>
<td>4.3 (5)</td>
<td>40.0</td>
<td></td>
</tr>
<tr>
<td>Tecolutlo</td>
<td>Male</td>
<td>45</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>--</td>
<td>Prefer Sc</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>40</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall</td>
<td>85</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2 Female preference for Sc phenotype in males. Results of paired sample t-tests comparing the average amount of time females associated with the Sc treatment and no Sc (mock) treatment across test days. Female preference data for Tanute, Cebolla, and Conchita populations taken from Chapter 3, which followed the identical protocol as the other three populations presented. SEM are in parentheses.

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>Time with Sc (SEM)</th>
<th>Time with non-Sc (SEM)</th>
<th>t</th>
<th>Prob.</th>
<th>Preference for Sc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tanute</td>
<td>19</td>
<td>496.8 (29.8)</td>
<td>289.5 (24.3)</td>
<td>4.26</td>
<td>P = 0.001</td>
<td>Prefer Sc</td>
</tr>
<tr>
<td>Cebolla</td>
<td>21</td>
<td>474.9 (30.1)</td>
<td>327.4 (23.6)</td>
<td>2.85</td>
<td>P = 0.01</td>
<td>Prefer Sc</td>
</tr>
<tr>
<td>Conchita</td>
<td>27</td>
<td>343.2 (27.4)</td>
<td>475.0 (28.2)</td>
<td>-2.41</td>
<td>P = 0.02</td>
<td>Prefer non-Sc</td>
</tr>
<tr>
<td>Chalpu.</td>
<td>25</td>
<td>418.4 (26.4)</td>
<td>442.4 (26.8)</td>
<td>-0.46</td>
<td>P = 0.65</td>
<td>No preference</td>
</tr>
<tr>
<td>San Martín</td>
<td>27</td>
<td>405.9 (26.3)</td>
<td>425.2 (30.5)</td>
<td>-0.35</td>
<td>P = 0.73</td>
<td>No preference</td>
</tr>
<tr>
<td>Tecolutlo</td>
<td>22</td>
<td>529.8 (32.7)</td>
<td>340.3 (29.9)</td>
<td>3.07</td>
<td>P = 0.006</td>
<td>Prefer Sc</td>
</tr>
</tbody>
</table>
Figure 4.1 Female preferences for Sc across populations. The Y axis represents the average strength of preference (± SEM) for the Sc phenotype in X. cortezi males across the six populations sampled which was calculated as the mean difference in the time females spent with the painted Sc male over the non-Sc male in the choice tests. Thus, positive values indicate females spent more time with Sc males and negative values signify that females spent more time with non-Sc male (i.e., discriminate against Sc). P values above the bars indicate if the average strength of preference for each population is significantly different than zero (one sample t-test). Pie charts underneath the bar graph depict the percentage of males and females with Xmrk (grey) in each population.
CONCLUDING REMARKS

The findings of this dissertation have several important implications for the *Xiphophorus* melanoma model. First, non-hybrid melanomas occur in more *Xiphophorus* species than initially realized. With increased sampling of different species and localities, I believe nonhybrid melanoma formation (as opposed to hybrid) will one day be the rule and not the exception. Second, the *Xmrk* oncogene can increase male aggression and thereby provides a competitive advantage for individuals in male-male competition. In addition, the macromelanophore patterns associated with the *Xmrk* oncogene can serve as signals in these male agonistic encounters. Third, female mate choice plays an important role in the evolutionary maintenance of this oncogene. Finally, the relative frequency of *Xmrk* within each sex of a population does influence female mating decisions and likely plays a factor in not only the continued polymorphism of *Xmrk* and Sc within *X. cortezi* but also other *Xiphophorus* that are polymorphic for *Xmrk*.

The *Xiphophorus* genus is a morphologically diverse group of fishes that has served a model system for studying sexual selection because numerous species incorporate various melanistic and color patterns as visual signals in mate choice and male-male competition. Melanoma research within *Xiphophorus* has also established this system as the premiere animal model for studying the genetic basis to skin cancer. This dissertation suggests that an interdisciplinary approach between these two fields of scientific investigation can be very insightful and informative. Narrowing the gap between these two fields is essential in furthering our understanding of the relationship between the phenotype and *Xmrk* genotype of this melanoma animal model.