Phylogenetic and Molecular Investigations of the Evolutionary Novelties, Sword and Gonopodium, in the Swordtail Fish (the genus *Xiphophorus*)

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Ji Hyoun Kang

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1. Referent: Prof. Axel Meyer, Ph.D.

2. Referent: Prof. Dr. rer. nat. Dr. h.c. Manfred Schartl

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General Introduction

Why are males animals generally more colorful or splendid than females? Why do males sometimes possess weapon-like structures? The answer to both of these questions might be "sexual selection". Sexual selection is known to be one of the key mechanisms for evolutionary phenotypic diversification and speciation (Andersson 1994). In particular, it has been suggested to be one of the major modes leading to the evolution of "novel" phenotypic traits that are not homologous to any given structures in ancestral species or the same organism (Müller & Wagner 1991). Several traits driven by sexual selection seen from nature have been recognized as evolutionary novelties that play an important role in female preference or male-male completion for mating success. My Ph.D. dissertation has aimed to understand the evolutionary history and the genetic basis of the evolutionary novel traits or sexually selected traits (male-specific characters) using phylogenetic and molecular approaches. To do so, I chose the genus *Xiphophorus* as a model, as it is one of the famous fish groups that have been studied to address many evolutionarily interesting questions about sexual selection and speciation such as female preference, evolutionary novelties, sexually selected traits, and hybridization (Kallman & Kazianis 2006).

The genus *Xiphophorus* (Family Poeciliidae) is a group of small live-bearing freshwater fish occurring from northern Mexico down to Belize and Honduras (Rosen 1960; Kallman & Kazianis 2006). Research efforts on this group of fish have increased our understanding of the genetic mechanisms that underlie evolutionary processes during speciation. *Xiphophorus* species are particularly interesting from an evolutionary perspective because of their unique morphological feature, the "sword". Some *Xiphophorus* species called swordtails, but not others, the platies, have a specific male trait, the "sword", that is an elongation and conspicuous coloration of the ventral rays of the caudal fin (Basolo 1990a, 1991). Questions for the origin and evolution of these swords grasp the attention of biologists since this trait is one of the rare, but unique examples from Darwin (1871) that sexual selection accounts for the evolution of exaggerated male traits that appear to be detrimental to their survival in nature. This trait has now become one of the textbook examples that is believed to be sexually selected by female choice (Basolo 1990a, 1995a, b). Another intriguing feature of this trait is its role of hybridization in speciation. Hybridization has been claimed as one of the major modes for the origin of new species. It has been suggested that

one of the swordtail species, *X. clemenciae,* originated through an ancient hybridization event between swordless platies and swordtail species (Meyer *et al.* 1994, 2006). Comprehensive phylogenetic investigations would be required to better understand those evolutionarily important questions such as interspecific phylogenetic relationships, the evolution of evolutionary novelty (sword), the mode of sexual selection and hybridization in the genus *Xiphophorus*. Despite several previous phylogenetic and behavioral studies that aimed to study the evolutionary history and ecological roles of the sword and gonopodium in determining mating success, less attention has been paid to the genetic basis underlying the development of these traits.

In **Chapter 1**, I reconstructed a comprehensive molecular phylogeny of all 26 known *Xiphophorus* species, including the four recently described species (*X. kallmani, X. mayae, X. mixei and X. monticolus*) to resolve the evolutionary relationships in this genus. This comprehensive molecular phylogeny of the entire genus suggests that in addition to *X. clemenciae*, one of the new species, *X. monticolus*, might be originated from an ancient hybridization event. Furthermore, the evolutionary history and character state analyses of the sword revealed that the sword was originated in the common ancestral lineage of this genus and lost again secondarily multiple times independently.

In the next two chapters, the focus has been turned to understand the genetic basis underlying evolutionarily interesting traits, the sword and gonopodium, in this fish group since the molecular mechanisms underlying the developmental processes of these features remain largely unknown. Interestingly, both the sword and the gonopodium can be hormonally induced. Therefore, androgen signaling and related target genes turned out to be good candidates to investigate the molecular developmental basis of those traits. First, we have focused on another male-specific trait, the gonopodium, found in the genus *Xiphophorus* and Poecillid fishes. The gonopodium is derived from the anal fin and formed by heavily modified rays 3, 4 and 5 that are transformed into this male intromittent organ used in copulation. This is an evolutionarily older trait than swords since gonopodia are found in all of the viviparous poeciliid fish family (evolutionary novelty in the family Poeciliidae).

In **Chapter 2**, I used a candidate gene approach to investigate whether androgen signaling and/or retinoic acid (RA) signaling are involved in the development of gonopodium (Rhinn & Dolle 2012). RA has been known as an important signaling molecule for paired appendage development in vertebrates (Morriss-Kay & Ward 1999). It also provides positional information along the proximodistal axis in limb development. Given that RA

signaling is required for the development of paired fins and fin regeneration, I explore the potential role of RA in the metamorphosis of an unpaired anal fin into the gonopodium in a green swordtail fish, *Xiphophorus hellerii*. I showed that *androgen receptor* is indeed activated and *retinaldehyde dehydrogenase* (*aldh1a2*), a RA synthesizing enzyme, and the retinoic acid receptors, *rar-ga* and *rar-gb* are expressed during the development of gonopodia. This is the first study showing that RA signaling pathways are activated in response to androgen signaling and that these pathways promote fin rays' growth and development during the metamorphosis of gonopodium development.

In **Chapter 3**, I extended the investigation of the genetic mechanisms underlying both sword and gonopodium developments into analyses at the whole transcriptome-level. Using genome-wide expression in these two traits by high-throughput transcriptome sequencing (RNA-Seq) in the swordtail, *Xiphophorus hellerii*, I aimed to better understand the architecture of gene regulatory networks of the development of these two evolutionary novelties under testosterone treatments. Large number of genes with tissue-specific expression patterns were identified. It was shown that a larger set of gene networks was co-opted during the development and evolution of the older gonopodium than in the younger, and morphologically less complex trait, the sword. This study provides a catalogue of candidate genes for future efforts to dissect the development of those sexually-selected exaggerated male traits in swordtails. Intriguingly, I found that several genes involved in the development of the sword are also commonly involved in male exaggerated ornaments across distantly related taxa.

Chapter 4 is a review with a comparative approach to unravel the "shared" genetic basis or genetic mechanisms of sexually selected traits or exaggerated male ornaments across diverse animal groups. The goals of this review are to list up reported genes or genetic networks involved in sexually selected traits in order to summarize emerging empirical data on the genetic mechanisms underlying sexually selected traits, and also to explore the common or shared genetic mechanisms behind those traits.

This dissertation is dedicated to understanding the evolutionary history of the sword by conducting a comprehensive phylogenetic investigation and exploring the molecular mechanisms underlying the evolutionarily important traits, sword and gonopodium, in *Xiphophorus hellerii*. These investigations would pave the future to get the complete picture of the development, evolution and the genetic basis of evolutionary novelties in this species.

Chapter 1. Comprehensive phylogenetic analysis of all species of swordtails and platies (Pisces: Genus *Xiphophorus*) uncovers a hybrid origin of a swordtail fish, *Xiphophorus monticolus*, and demonstrates that the sexually selected sword originated in the ancestral lineage of the genus, but was lost again secondarily

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Ji Hyoun Kang[1,2]
Email: jihyoun.kang@uni-konstanz.de

Manfred Schartl[3]
Email: phch1@biozentrum.uni-wuerzburg.de

Ronald B Walter[4]
Email: rwalter@txstate.edu

Axel Meyer[1,2*]
[*] Corresponding author
Email: axel.meyer@uni-konstanz.de

[1] Lehrstuhl für Zoologie und Evolutionsbiologie, Department of Biology, University of Konstanz, Universitätsstraße 10, Konstanz 78457, Germany

[2] Konstanz Research School Chemical Biology, University of Konstanz, Konstanz, Germany

[3] Physiological Chemistry, Biozentrum, University of Würzburg, Am Hubland, Würzburg 97074, Germany

[4] Department of Chemistry and Biochemistry, Texas State University-San Marcos, 601 University Dr, San Marcos, TX 78666, USA

Abstract

Background

Males in some species of the genus *Xiphophorus*, small freshwater fishes from Meso-America, have an extended caudal fin, or sword – hence their common name "swordtails". Longer swords are preferred by females from both sworded and – surprisingly also, non-sworded (platyfish) species that belong to the same genus. Swordtails have been studied widely as models in research on sexual selection. Specifically, the pre-existing bias hypothesis was interpreted to best explain the observed bias of females in presumed ancestral lineages of swordless species that show a preference for assumed derived males with swords over their conspecific swordless males. However, many of the phylogenetic relationships within this genus still remained unresolved. Here we construct a comprehensive molecular phylogeny of all 26 known *Xiphophorus* species, including the four recently described species (*X. kallmani*, *X. mayae*, *X. mixei* and *X. monticolus*). We use two mitochondrial and six new nuclear markers in an effort to increase the understanding of the evolutionary relationships among the species in this genus. Based on the phylogeny, the evolutionary history and character state evolution of the sword was reconstructed and found to have originated in the common ancestral lineage of the genus *Xiphophorus* and that it was lost again secondarily.

Results

We estimated the evolutionary relationships among all known species of the genus *Xiphophorus* based on the largest set of DNA markers so far. The phylogeny indicates that one of the newly described swordtail species, *Xiphophorus monticolus*, is likely to have arisen through hybridization since it is placed with the southern platyfish in the mitochondrial phylogeny, but with the southern swordtails in the nuclear phylogeny. Such discordance between these two types of markers is a strong indication for a hybrid origin. Additionally, by using a maximum likelihood approach the possession of the sexually selected sword trait is shown to be the most likely ancestral state for the genus *Xiphophorus*. Further, we provide a well supported estimation of the phylogenetic relationships between the previously unresolved northern swordtail groups.

Conclusions

This comprehensive molecular phylogeny of the entire genus *Xiphophorus* provides evidence that a second swordtail species, *X. monticolus*, arose through hybridization. Previously, we demonstrated that *X. clemenciae*, another southern swordtail species, arose via hybridization. These findings highlight the potential key role of hybridization in the evolution of this genus and suggest the need for further investigations into how hybridization contributes to speciation more generally.

Background

Species in the genus *Xiphophorus* (Family Poeciliidae) are small live-bearing freshwater fish that are distributed from northern Mexico to Belize and Honduras (Rosen 1960; Kallman & Kazianis 2006). Poeciliids have been widely studied in fields ranging from ecology, evolution, genetics, and genomics to systematics (Rosen 1979). These fish have been investigated in an effort to improve our understanding of the evolution of several life-history and behavioral traits including viviparity (Schrader & Travis 2008, 2009), the placenta (Meredith *et al.* 2010) and female mating preference for exaggerated male traits such as the sword. The sexually selected sword trait is generally assumed to have arisen through "sensory exploitation" and a "pre-existing bias" (Basolo 1990b, 1995b). More recently, fish of this group have also been the foci of studies aimed at uncovering the genetic mechanisms underlying evolutionary processing during speciation (Haskins *et al.* 1950; Lindholm & Breden 2002; Meyer *et al.* 2006; Rosenthal & Garcia de Leon 2006; Ward *et al.* 2008; Plath *et al.* 2010; Rosenthal *et al.* 2011).

The genus *Xiphophorus* is particularly interesting from an evolutionary perspective because several of its species have a unique morphological feature, the "sword". The "sword-bearing" species of *Xiphophorus*, are called swordtails, and the others, that lack the sword, are called platyfish. Their males lack this male specific trait - a conspicuously colored elongation of the ventral rays of the caudal fin (Basolo 1990a, 1991). The investigation of this unique feature has provided many interesting evolutionary insights, particularly study in terms of sexual selection. The evolution of this exaggerated male trait appears to be detrimental to the males' survival, since, although it is attractive to females, it also makes them more conspicuous to predators (Darwin 1871).

The pre-existing bias hypothesis (Ryan 1990, 1998) was proposed to explain best the evolution of the sword (Basolo 1990a, 1995b). This is based on a traditional phylogeny of the genus that places the platies basal to the more derived swordtails and laboratory choice experiments that showed that platy females prefer heterospecific sworded males over their non-sworded conspecific males (Basolo 1990a, 1995b). The traditional phylogenetic hypothesis therefore suggested that the females' preference for the sword arose before the trait itself, and hence, the female preference might have driven the subsequent evolution of the males' trait. The pre-existing bias hypothesis relies on an explicit phylogenetic hypothesis and can therefore be tested (Rosen 1979; Rauchenberger *et al.* 1990).

However, recent molecular phylogenetic studies suggested that the swordless platy species may instead be more derived than the more basally-placed sworded lineages (Meyer *et al.* 1994, 2006). This tree topology called the applicability of the pre-existing bias hypothesis for the evolution of the sword into question since the reconstruction of the evolution of the sword based on the molecular phylogeny suggested that the sword originated in the ancestor of this genus and was lost repeatedly and independently during the evolutionary history of this genus (Meyer *et al.* 1994, 2006). This topology further suggests that the females' bias for swords might have been retained in the derived, but non-sworded platyfish species (Basolo 1990a, 1990b, 1995a). But subsequent testing of female preferences for swords among poeciliid species outside the genus *Xiphophorus*, namely of *Priapella*, showed that females of these species also preferred sworded males (Basolo 1995b). Since *Priapella* is one of the closest genera to *Xiphophorus* (Hrbek *et al.* 2007) and *Priapella*, just as all other poeciliid males do not have swords, their females' preference for swords, would tend to lend support again to the pre-existing bias hypothesis as the best explanation for the initial evolution of the sword.

Obviously, the correct phylogeny for the genus is important for the inferred history of the sword. Several previous studies have performed analyses of ancestral state reconstruction of the sword in the genus *Xiphophorus* to understand its evolutionary history and to test for the pre-existing bias hypothesis. Some studies differed from each other in terms of how the sword was scored since some species are polymorphic in length or coloration of the sword. This, as well as whether parsimony or maximum likelihood was used, could somewhat alter results of the ancestral state reconstruction (Wiens & Morris 1996; Meyer *et al.* 1997; Schluter *et al.* 1997). Based on the molecular phylogeny "sworded" was inferred to be the ancestral condition for all *Xiphophorus* species when caudal extension (of any length) was considered a sword (Wiens & Morris 1996; Meyer 1997) whereas its ancestral state was inconsistent - when short extension was assigned to another state (i.e. protrusion) (Meyer 1997). Wiens and Morris (Wiens & Morris 1996) argued that uncolored "protrusion" should not be scored as a sword since the pre-existing bias was demonstrated through female preference for colored caudal extension (Basolo 1990a). They also demonstrated that "swordless" is an ancestral condition in their parsimony analysis supporting the pre-existing bias for the evolutionary origin of the sword. However, a likelihood reconstruction using the same description of the sword (i.e., colored extension) favored by Basolo (Basolo 1990a, b) and Wiens and Morris (Wiens & Morris 1996), again resulted in an uncertain ancestral state (Schluter *et al.* 1997). Since the evolution of female preferences for swords has become a textbook example for the pre-existing bias hypothesis we, therefore, revisited this issue here

based on the most comprehensive phylogeny, so far, most comprehensive, both in terms of taxa and markers.

Many of the previous phylogenetic analyses of this genus have been conducted solely (or at least mostly) based on either mitochondrial or morphological characters and the recently described four new species [*X. kallmani* (Meyer & Schartl 2003), *X. mayae* (Meyer & Schartl 2002), *X. mixei* and *X. monticolus* (Kallman *et al.* 2004)] were not included in any phylogenetic analysis so far. Since ancestral state reconstructions need to be performed based on the most comprehensive phylogeny using different sword descriptions and different reconstruction methods (e.g., parsimony and likelihood) to understand the origin of the sword more clearly we set out to do this here.

Hybridization has been claimed to be one of the major modes for the origin of new species in some evolutionary lineages (Arnold 1997; Arnold & Meyer 2006; Mallet 2007) and natural hybridization events between distinct populations or closely related taxa have been reported in various plants and animal taxa [e.g., (Grant & Grant 2002; Seehausen 2004; Arnold & Meyer 2006; Mahe *et al.* 2007; Rosenthal *et al.* 2008; Aboim *et al.* 2010; Culumber *et al.* 2011)]. Introgressive hybridization has been observed also in some lineages of freshwater fishes, for instance, whitefish (Lu *et al.* 2001), Lake Tanganyikan cichlids (Salzburger *et al.* 2002; Koblmuller *et al.* 2007) and cyprinid fish (Aboim *et al.* 2010). Hybrid speciation by comparison, has been documented only rarely (Mallet 2007). The role of natural hybridization in speciation is still debated due to the general observation of decreased fitness and sterility of hybrids (Arnold 1997; Barton 2001).

It turns out that *Xiphophorus* fish are an excellent model system for examining the role of hybridization in speciation since we previously discovered that one species of this genus might be of hybrid origin (Meyer *et al.* 1994, 2006). Discordance between different types of molecular markers is routinely recognized as evidence for hybridization events, and such discordance has been uncovered, for example, in flies (DeSalle & Giddings 1986), goats (Ropiquet & Hassanin 2006), leaf monkeys (Ting *et al.* 2008) and vipers (Barbanera *et al.* 2009). Previously, Meyer *et al*. (Meyer *et al.* 1994, 2006) found a discrepancy in the placement of the swordtail species, *X. clemenciae*, in mitochondrial versus nuclear marker based phylogenetic trees. *Xiphophorus clemenciae*, a southern swordtail, grouped with the southern swordtails in the nuclear phylogeny (Meyer *et al.* 2006), but was assigned to the southern platyfish lineage in the mitochondrial phylogeny. Meyer *et al*. (Meyer *et al.* 2006) suggested that *X. clemenciae* originated in a relatively ancient hybridization event between a swordless female platyfish from a geographically widespread lineage such as *X. maculatus*,

and a similarly widespread southern swordtail species, such as *X. hellerii*. Additional lines of evidence, including laboratory mate choice trials, the intermediate length of the sword in *X. clemenciae* and artificially produced hybrids relative to the two putative close relatives of the parental species, *X. maculatus* and *X. hellerii* (Meyer *et al.* 2006), further support the hypothesis of a hybrid origin of *X. clemenciae*. Interestingly, on-going hybridization has been reported to occur between the northern swordtails *X. malinche* and *X. birchmanni* (Culumber *et al.* 2011; Rosenthal *et al.* 2003) and hybrids can be produced under laboratory conditions for most species in this genus (Gordon 1947; Siciliano *et al*. 1971; Ozato & Wakamatsu 1981; Kazianis *et al.* 1996).

Although the origin and evolution of the sword (Basolo 1990a, 1995a; Meyer 1997, Zauner *et al*. 2003; Wilkins 2004; Morris *et al*. 2005; Eibner *et al*. 2008) and the role of hybridization in the genus *Xiphophorus* (Kallman & Kazianis 2006) have been addressed before, some of the phylogenetic relationships in this genus still remained uncertain. Traditionally, the genus *Xiphophorus* has been suggested to consist of four major lineages based on their geographical distributions and other phenotypic traits (i.e., northern platyfish, northern swordtails, southern platyfish and southern swordtails; Figure 1.1a) (Rauchenberger *et al.* 1990; Meyer *et al.* 1994, 1996; Kallman & Kazianis 2006). The monophyly and the relationships among those four lineages are not consistently supported in phylogenetic studies using molecular or combined molecular and morphological traits. For example, it has been difficult to assign *X. andersi* (Meyer & Schartl 1980) to any specific lineage and inconsistent phylogenetic placements were found based on morphological characters and molecular based phylogenetic analyses (Meyer *et al.* 1994, 2006). *Xiphophorus andersi* has some platy as well as some swordtail features – it is an elongated – swordtail-like – species, but lacks the pronounced, colored ventral extension of the caudal fin. Also, geographical distributions of some species are inconsistent with those of other members of the lineages to which they were assigned; for example, a southern platyfish, *X. xiphidium* occurs further north than the northern swordtails (Kallman & Kazianis 2006) (see Figure 1.1a).

The northern swordtail lineage has received much attention from researchers because of its remarkable diversity in sexual and ecological traits (Morris *et al.* 1995; McLennan & Ryan 1997; Cummings *et al.* 2003; Morris *et al*. 2005), but the phylogenetic relationships among some of its nine described species remain incompletely resolved as well, hindering the interpretation of data in a phylogenetic context. Rauchenberger *et al*. (Rauchenberger *et al.* 1990) presented a comprehensive phylogeny using morphology, pigmentation and electrophoretic characters and suggested that there are three clades within this group – the

montezumae clade (*X. nezahualcoyotl*, *X. continens* and *X. montezumae*), the *pygmaeus* clade (*X. nigrensis*, *X. multilineatus* and *X. pygmaeus*) and the *cortezi* clade (*X. cortezi*, *X. birchmanni* and *X. malinche*). However, these clades have not been consistently supported in subsequent phylogenetic studies using morphology, molecular data or a combination of those (e.g., pigmentation, allozyme, RAPD [Random Amplified Polymorphic DNA], mtDNA and nuclear DNA) (Rauchenberger *et al.* 1990; Meyer *et al.* 1994, 2006; Borowsky *et al.* 1995; Marcus & McCune 1999; Morris *et al*. 2001; Gutierrez-Rodriguez *et al.* 2007; Gutierrez-Rodriguez *et al.* 2008).

Recently, four additional species have been described in this genus: *X. kallmani*, *X. mayae*, *X. mixei* and *X. monticolus* (Figures 1.1a, b). All these four new species are southern swordtails based on their geographical origins and phenotypic characteristics (Kallman & Kazianis 2006). Yet, their molecular phylogenetic relationships to the other *Xiphophorus* species have not been examined so far.

Here, we conduct a comprehensive molecular phylogenetic analysis of the genus *Xiphophorus* that includes also these four newly described species. By using more informative nuclear markers, we aim to provide a better understanding of the phylogeny of this entire genus, its evolutionary history, and the evolution of the sword. We discovered that one of the newly described species, *X. monticolus*, is likely to have originated from an ancient hybridization event, as we found *X. clemenciae* to be the case before.

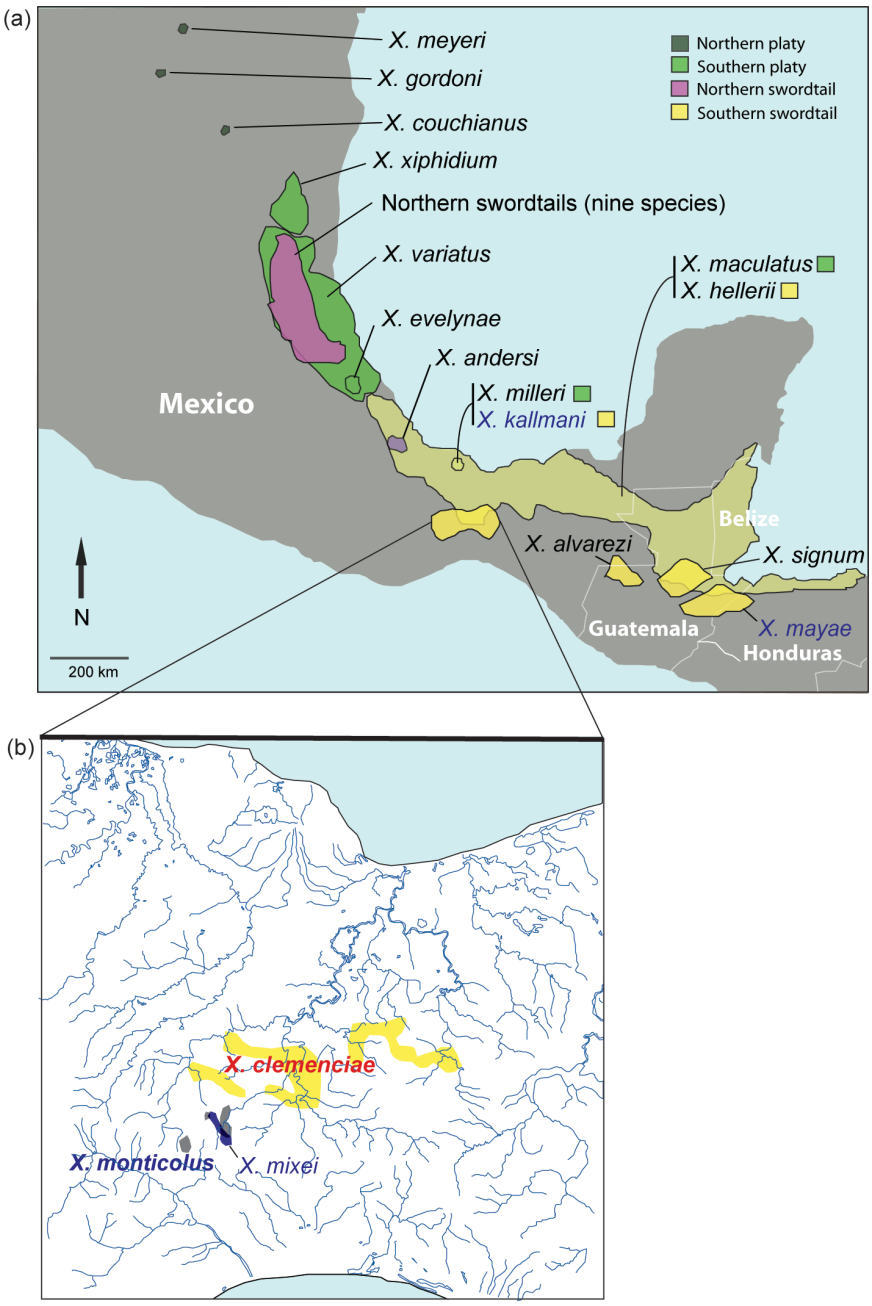

Figure 1.1 Map of the distributions of *Xiphophorus* species.

(a) Geographical distributions of all described 26 species in the genus *Xiphophorus* including the four newly described species – *X. monticolus*, *X. mixei*, *X. kallmani* and *X. mayae* (colored in blue) and two species of a putatively hybrid origin, *X. monticolus* and *X. clemenciae* (in bold). (b) Geographical distributions of three species in the *clemenciae* clade (maps are modified from (Kallman & Kazianis 2006; Jones *et al*. 2012)).

Results

Phylogenetic analyses

We reconstructed the phylogenetic relationships of the genus *Xiphophorus*, including four newly described species, using four different methods [i.e., BI (Bayesian Inference), ML (Maximum-Likelihood), NJ (Neighbor-Joining), MP (Maximum Parsimony)]. Two mitochondrial (cytochrome *b* and control region) and eleven nuclear loci [recombination activating gene 1 (Rag 1)/exon 3, tyrosine kinase (X-*src*), three non-coding flanking regions of the microsatellite loci (D2, D8 and T36) (Meyer *et al.* 1994; Seckinger *et al.* 2002; Meyer *et al.* 2006), guanine nucleotide-binding protein (G protein) subunit gamma13 (GNG 13), glucose-6-phosphate dehydrogenase (G6PD, 6th intron), Uracil-DNA-glycosylase (UNG, 4th intron), DNA polymerase beta (POLB, 7th to 11th intron), flap structure-specific endonuclease 1 (FEN1, 3rd intron) and tumor protein p53 (TP53, 4th intron)] were used for phylogenetic analyses. Since mitochondrial and nuclear DNA have different evolutionary histories, mitochondrial and nuclear phylogenetic trees were separately reconstructed. The total lengths of the aligned sequences used for the mitochondrial and nuclear phylogenies were 1239 bp and 7276 bp, respectively; of which 291 (218; without outgroup) nucleotide sites were variable and 192 (120) of those were parsimony informative for the mitochondrial loci, whereas 690 (499) nucleotide positions were variable and 412 (247) of those were informative for the nuclear loci. In addition, we reconstructed the phylogeny using a combination of the mitochondrial and nuclear data (8515 bp) to provide an overall view of evolutionary relationships of *Xiphophorus* using all data (Additional file 1). This combined tree showed nearly identical phylogenetic relationships among the major lineages (i.e., northern platyfish, northern swordtails, southern platyfish and southern swordtails) with the nuclear tree. Two species, *Priapella compressa* and *P. olmecae*, were selected as outgroups considering previously published phylogenies of the family Poeciliidae (Hrbek *et al.* 2007; Doadrio *et al.* 2009) as well as our recently reconstructed poeciliid phylogeny (Kang and Meyer, unpublished data). Both previous poeciliid phylogenies (Hrbek *et al.* 2007; Doadrio *et al.* 2009) independently support several different species as closely related taxa to *Xiphophorus*, although with very low support and conflicting relationships. Our recent poeciliid phylogeny (Kang and Meyer, unpublished data), which is based on several mitochondrial and nuclear DNA markers combined (7942 bp) and is the largest data set so far and provides support for the genera *Heterandria* and *Priapella* being the most closely related

taxa to *Xiphophorus*; however, *Heterandria* showed a longer branch than *Priapella* in the phylogeny, which is also consistent with a recent RAD-marker based *Xiphophorus* phylogeny from the Meyer laboratory (Jones *et al.* 2013). Genetic diversity indices and evolutionary models for each locus are shown in Table 1.1.

Mitochondrial phylogeny

The phylogeny based on the mtDNA markers placed the northern swordtails as the sister group to the clade formed by southern swordtails and platyfish (Figure 1.2a), which is consistent with previous mitochondrial phylogenies (Meyer *et al.* 1994, 2006). But the sister group relationship between the platyfish and the southern swordtails was supported by only moderate bootstrap values (51–85) in all phylogenetic estimations (i.e., BI, ML, NJ, MP) (Figure 1.2a).

Whereas *Xiphophorus monticolus*, although clearly phenotypically a southern swordtail, was placed with the southern platy group, the other three newly described species (*X. kallmani*, *X. mayae* and *X. mixei*) were placed in the southern swordtail clade. The monophyly based on mtDNA of the southern swordtails, except *X. clemenciae* and *X. monticolus*, was supported with high Bayesian posterior probabilities (100) and quite convincing bootstrap values (80–96), but the phylogenetic relationships within this southern swordtail group could not be resolved with high phylogenetic confidence.

The monophyly of the northern swordtail lineage was supported, albeit only with moderate bootstrap values (73–85), but the phylogenetic positions of *X. birchmanni* and *X. pygmaeus* were not consistent among the four different phylogenetic analysis methods. Overall, the recovered phylogeny of the 26 species of *Xiphophorus* was almost identical to our previous 22-taxa mtDNA-phylogeny (Meyer *et al.* 2006).

Table 1.1 Genetic diversity indices from two mitochondrial and eleven nuclear loci examined in this study.

Name	Locus	Nucleotides (bp)	Variable sites	Parsimony - informative sites	*p*-distance	SE	Model of evolution
Nuclear	Combined	**7276**	**690**	**412**	**0.017**	**0.001**	**TVM+G**
D2	Flanking region of the microsatellite loci D2	393	55	34	0.028	0.004	TIM3+G
D8	Flanking region of the microsatellite loci D8	516	59	37	0.019	0.003	TPM2uf+G
T36	Flanking region of the microsatellite loci T36	394	45	33	0.024	0.004	HKY
X-*src*	Tyrosine kinase	520	66	45	0.024	0.003	TVM+I
Rag1	Recombination activating gene	1574	64	40	0.007	0.001	TIM3+G
GNG13	Guanine nucleotide binding protein (G protein) subunit gamma 13 (1^{st} intron)	531	46	33	0.017	0.003	TPM2uf
G6PD	Glucose-6-phosphate dehydrogenase (6^{th} intron)	526	48	27	0.018	0.003	HKY+G
UNG	Uracil-DNA-glycosylase (4^{th} intron)	277	18	10	0.011	0.003	JC+G
POLB	DNA polymerase beta (7^{th} to 11^{th} intron)	672	43	22	0.01	0.002	TPM3uf+G
FEN1	Flap structure-specific endonuclease 1 (3^{rd} intron)	827	123	63	0.021	0.003	TPM3uf+G
TP53	Tumor protein p53 (4^{th} intron)	1046	123	68	0.024	0.003	TPM1uf+G
mtDNA	Combined	**1239**	**291**	**192**	**0.051**	**0.003**	**TPM1uf+I+G**
cyt*b*	Cytochrome *b*	360	112	67	0.059	0.006	TPM1uf+I+G
D-loop	Control region	879	179	125	0.047	0.004	TIM2+I+G

Nucleotide diversity (the average of *p*-distance between all the species) and standard error (SE) estimate were calculated using MEGA 4.0 (Tamura *et al.* 2007). Nucleotide diversity indicates estimates of average sequence divergence across all sequence-pairs. SE was estimated by a bootstrap procedure (1000 replicates). The best-fit evolutionary model was selected for each gene as well as for combined entire sequences of mitochondrial and nuclear genes using jModeltest under the Akaike Information Criterion (Posada 2008).

Nuclear phylogeny

The phylogeny based on eleven nuclear loci (see Table 1.1) provided good evidence for the monophyly of the platyfish plus *X. andersi* with high Bayesian posterior probabilities (100) and high bootstrap values (98) for maximum likelihood (Figure 1.2b). The monophyly of the northern platies was invariably strongly supported in all types of phylogenetic inferences, whereas the monophyly of the southern platyfish was not supported (Figure 1.2b).

The monophyly of the northern swordtails was strongly supported by all phylogenetic analyses (Figure 1.2b), whereas the southern swordtails were resolved as paraphyletic in some analyses. We found that the position of the two major lineages of swordtails in relation to the platies was differently resolved between the nuclear DNA and mtDNA phylogenies – and hasten to note, that the alternative topologies were relatively weakly supported only. Specifically, the platies (northern and southern) were more closely related to the southern swordtails than the northern swordtails in the mtDNA phylogeny, whereas the northern swordtail clade was identified as the sister group to the platies in the nuclear DNA phylogeny (Figure 1.2b).

Although the monophyly of the northern swordtails was strongly supported by all phylogenetic analyses (Figure 1.2b), within the northern swordtails, only two clades (the *montezumae* lineage and the *pygmaeus* lineage) were well-supported. Both sets of markers supported the monophyly of the northern swordtails, however, internal relationships were not clearly resolved in whole species phylogenies.

Contrary to the mtDNA-based tree, all four newly described species were grouped with previously recognized southern swordtail species (Kallman & Kazianis 2006; Meyer *et al.* 2006). Our data provide high support for both the *clemenciae* and *hellerii* clades (see also Kallman *et al.* 2004) including all the newly described species in all phylogenetic methods (Figure 1.2b). Two of the newly described species, *X. mixei* and *X. monticolus*, group together with *X. clemenciae* (*clemenciae* clade), whereas the other two new species, *X. mayae* and *X. kallmani*, group with the remaining southern swordtails including *X. hellerii* (*hellerii* clade) (Figure 1.2b).

The analyses of the nuclear DNA data suggest that the *hellerii* clade is basal to all other swordtails and platies, and – tentatively – the *clemenciae* clade is the sister group to the northern swordtails plus the platies. While BI and ML (but with only 64 % bootstrap support) methods suggest that the southern swordtails are paraphyletic, NJ and MP methods support

their monophyly with bootstrap values of 77 and 73, respectively. We stress that the hypothesis of monophyly of the southern swordtails could not be rejected [P = 0.472, Approximately Unbiased (AU) test; P = 0.965, Shimodaira-Hasegawa (SH) test] and we have a very large RADseq data set (Jones *et al.* 2013) that also supports the monophyly of southern swordtails and their basal placement in the genus as sister to the platies+northern swordtails. Therefore, we continue to regard the monophyly of the southern swordtails to be more strongly supported. Our RADseq (restriction site-associated DNA sequencing) data set (Jones *et al.* 2013) on this issue provides the strongest phylogenetic support yet for the monophyly of the southern swordtails based on a data set of about 66,000 SNPs. It remains an open issue why these four phylogenetic methods suggest a different basal node for the genus based on mtDNA and nuclear data sets.

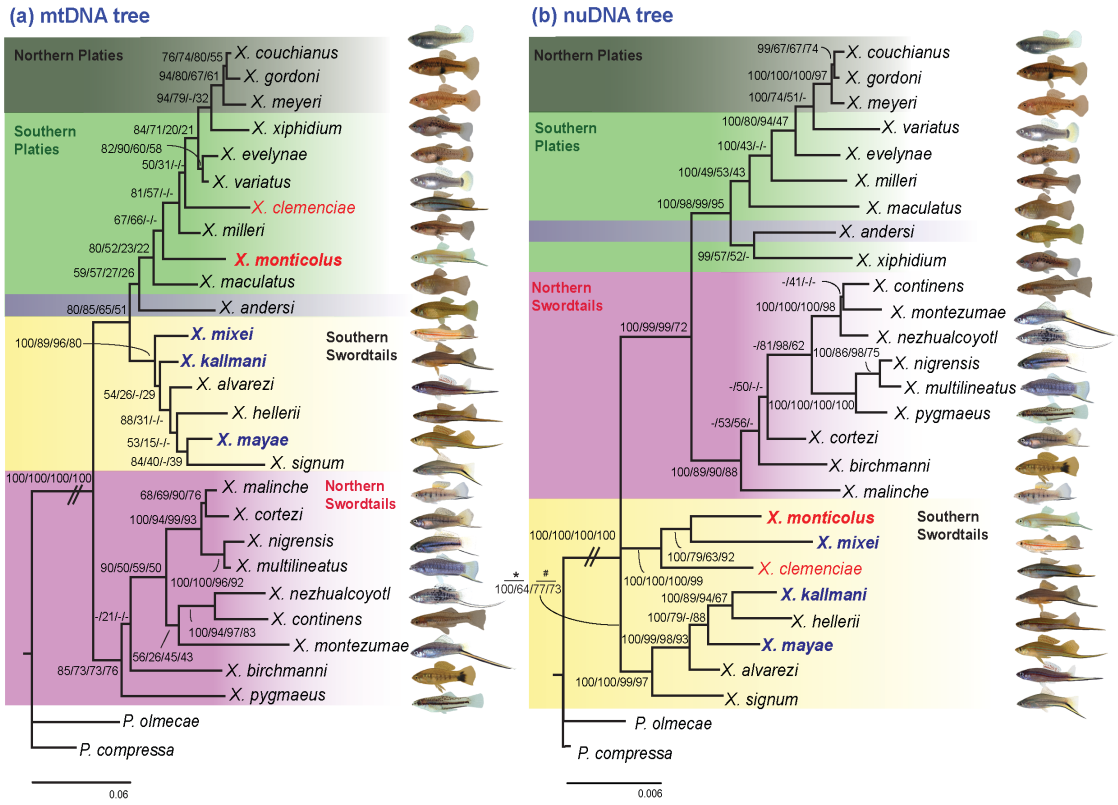

Figure 1.2 Mitochondrial and nuclear phylogenies of all 26 *Xiphophorus* species.

The phylogenetic trees were constructed from (a) combined sequences of two mtDNA loci (1239 bp) (complete control region and a segment of the cytochrome *b* gene) and (b) combined sequences of eleven nuclear loci (7276 bp). We indicate with (*) and (#) the supporting values of monophyly and paraphyly of the southern swordtails respectively. Numbers indicate Bayesian posterior probabilities, Maximum-Likelihood, Neighbor-Joining and Maximum-Parsimony bootstrap values, respectively. The values of the branch length that was truncated are 0.447 (a) and 0.038 (b). The two hybrid origin species – *Xiphophorus monticolus*, one of the four newly described species, and *X. clemenciae* are highlighted in red and the three remaining new species, *X. mixei*, *X. kallmani* and *X. mayae* in blue. Some fish images were obtained from the *Xiphophorus* Genetic Stock Center (Texas) with permission.

Discrepancy between mtDNA and nuclear DNA phylogenies: indication for a hybrid origin of *Xiphophorus monticolus*

Our extended phylogenetic analyses, including six new nuclear loci (3879 bp) and four new species, now provide evidence for the hypothesis of a hybrid origin of two *Xiphophorus* species, *X. clemenciae* and *X. monticolus*. Incongruence was found in the placement of both of these southern swordtail species between the mitochondrial and nuclear phylogenies (Figure 1.2) in that mitochondrially those two species were placed among the southern platies and, based on nuclear DNA sequences, these two species of southern swordtails were resolved to be part of the southern swordtail clade – a rather distant lineage of the genus they clearly belong to phenotypically. These results confirm the previously reported discrepancy in the placement of *X. clemenciae* (Meyer *et al.* 1994, 2006) and suggest that an additional species, *X. monticolus*, also arose through similar mechanisms.

Therefore, the observed incongruence was further analyzed to determine whether the mtDNA phylogeny is indeed different from the nuclear tree with respect to the positions of *X. monticolus* and *X. clemenciae*. We compared both best mitochondrial and nuclear ML unconstrained trees (Figure 1.2) with their best ML constrained trees. In the mitochondrial data set, the best ML unconstrained tree (Figure 1.2a) was strongly favored in comparison to the constrained tree, which placed: 1) *X. monticolus* with *X. mixei* and 2) *X. monticolus* with *X. mixei* and *X. clemenciae*, similar to the nuclear tree (unconstrained tree; $P = 1.00$, constrained tree; $P < 0.05$, AU and SH tests; Table 1.2) (Shimodaira 2002). In the nuclear data set, the best ML unconstrained tree (Figure 1.2b) was significantly better at "explaining" the nuclear data set than the best ML constrained tree, which assigned to 1) *X. monticolus* with the platies or 2) *X. monticolus* with the platies, apart from *X. maculatus* (unconstrained tree; $P = 1.00$, constrained tree; $P < 0.001$, AU and SH tests; Table 1.2). Both phylogenetic hypotheses that place *X. clemenciae* and *X. monticolus* with the platies in the nuclear phylogeny, and that group these species with the southern swordtails in the mitochondrial phylogeny were strongly rejected ($P < 0.05$, AU and SH tests; Table 1.2).

If *X. monticolus* arose by hybridization, closely related extant taxa might be genetically close to the putative maternal and paternal species. In the mitochondrial phylogeny, *X. monticolus* is closely related to platies such as *X. evelynae*, *X. variatus*, *X. milleri* and *X. maculatus* with 1.9 %, 2.3 %, 2.5 % and 2.9 % sequence divergence, respectively. In the nuclear phylogeny, *X. monticolus* was grouped however, with *X. clemenciae* and *X. mixei* (with 0.9 % and 1.1 % of sequence divergence only, respectively).

The taxa most genetically similar to *X. monticolus*, apart from the *clemenciae* clade (*X. clemenciae*, *X. mixei* and *X. monticolus*), in the nuclear tree were *X. mayae* and *X. signum* with 1.1 % and 1.2 % sequence divergence. These data provide some hints as to the timing, species identity of the maternal and paternal lineages, and phylogeography of the hybridization event (see below).

Table 1.2 Comparison of the best ML unconstrained trees with the constrained best ML trees of both mitochondrial and nuclear phylogenies.

Mitochondrial DNA	**AU (SE)**	**SH (SE)**	**Nuclear DNA**	**AU (SE)**	**SH (SE)**
Unconstraint best ML tree (Figure 1.2a)	0.992 (0.001)	0.989 (0.001)	Unconstraint best ML tree (Figure 1.2b)	1 (0)	1(0)
Constraint best ML tree (*X. monticolus* with *X. mixei*)	0.010 (0.002)	0.022 (0.001)	Constraint best ML tree (*X. monticolus* with platies)	7.00E-005(0)	1.00E-04(0)
Constraint best ML tree (*X. monticolus* with *X. mixei* and *X. clemenciae*)	0.001 (0)	0.001 (0)	Constraint best ML tree (*X. monticolus* with platies except *X. maculatus*)	2.00E-058(0)	0(0)

P-values were estimated by AU [Approximately Unbiased] and SH [Shimodaira-Hasegawa] tests implemented in CONSEL (Shimodaira & Hasegawa 2001) (SE: standard error).

Northern swordtail phylogeny

The relationships among the nine northern swordtail species differed more between the mtDNA and nuclear DNA phylogenies (Figure 1.2). Because species of the northern swordtail lineage are used by several laboratories for behavioral ecological work and as model for study of evolutionary questions, we, therefore, conducted additional analyses on the northern swordtails only - based on the nuclear and mitochondrial data sets separately and also combined both data sets (Figure 1.3) – in an effort to resolve the phylogenetic relationships among the species in this lineage. For these analyses, two platyfish species (*X. evelynae* and *X. gordoni*) were used as outgroups (Figure 1.3). Aligned nucleotide sequences of the mitochondrial loci contained 151 variable sites and 85 of those were parsimony informative with 0.041 [SE (Standard Error) = 0.003] of average *p*-distance, whereas the nuclear loci exhibited 245 variable sites and 110 of those were informative with 0.01 (SE = 0.001). The best-fit evolutionary models chosen (jModeltest 0.1.1, (Posada 2008)) for the mitochondrial and nuclear loci were TPM1uf+I+G and TPM1uf+G, respectively. The combined mitochondrial and nuclear alignments contained 396 variable sites and 195 of these

were parsimony informative with 0.015 (SE = 0.001) of average *p*-distance and TrN+G was determined as the best-fit evolutionary model.

Analyses of the northern swordtails based on the nuclear and mitochondrial separate data sets (Figures 1.3a, b) revealed almost identical tree topologies compared to the whole nuclear and mitochondrial phylogenies with all species (Figures 1.2a, b). Nevertheless, those analyses provided much higher bootstrap values and all four different phylogenetic estimations (BI, ML, NJ and MP) agree on the majority of nodes. In the nuclear phylogeny (Figure 1.3b), we recovered the *pygmaeus* and *montezumae* clades, but not the *cortezi* clade, which is consistent with several previous studies using different markers and morphological characters (Borowsky *et al.* 1995; Rauchenberger *et al.* 1990; Marcus & McCune 1999; Morris *et al*. 2001; Meyer *et al.* 2006). The mitochondrial and nuclear combined data set (Figure 1.3c) showed similar phylogenetic relationships to the nuclear phylogeny, and still could not recover the *cortezi* clade. In the mitochondrial phylogeny (Figure 1.3a) however, some phylogenetic relationships are incongruent with the nuclear phylogeny. For example, two strongly supported clades (*X. montezumae* and *X. pygmaeus* clades) in the nuclear phylogeny were not recovered in the mtDNA analyses. Here *X. birchmanni* might be basal to all other northern swordtails, but this remains unresolved in the nuclear phylogeny. The clade of *X. nigrensis* and *X. multilineatus*, which was strongly supported in our mtDNA, nuclear and combined phylogenetic analyses, and also previous studies (Rauchenberger *et al.* 1990; Morris *et al*. 2001; Meyer *et al.* 2006), is grouped with *X. cortezi* and *X. malinche* in the mitochondrial DNA phylogeny, whereas it is more closely related to *X. pygmaeus* in the nuclear DNA phylogeny. These better resolved mitochondrial and nuclear phylogenetic analyses clearly show many incongruent phylogenetic positions between two types of molecular marker based phylogenies in the northern swordtails.

Para- or monophyly of the southern swordtails

We found conflicting support for monophyly (100% Bayesian and 64% ML) or paraphyly (77% NJ and 73% MP) of the southern swordtails based on the four phylogenetic methods used for the nuclear markers (Figure 1.2b and see combined tree Additional file 1). But, the hypothesis of monophyly could not be rejected (see above) and was very strongly supported by our unpublished RADseq data set (Jones *et al.* 2013). To further investigate the phylogenetic relationships among the southern swordtails, we compared the topology of trees constructed using each gene individually (Additional file 2). Two classes of genes suggest

different evolutionary hypotheses regarding monophyletic or paraphyletic relationship of the *clemenciae* and *hellerii* clades. Seven nuclear loci (D8, X-*src*, Rag1, GNG13, G6PD, POLB and FEN1) support a paraphyletic relationship of the southern swordtails, but three loci (UNG, TP53, T36) show monophyly and one locus (D2) could not show their relationshipclearly (Additional file 2). Further phylogenetic analyses based on the combined set of those seven markers inferred the paraphyly (Additional file 3a) and four markers (UNG, TP53, T36 and D2) supported the monophyly of the southern swordtails (Additional file 3b) with higher bootstrap values than when the eleven markers were combined. Clearly, there are partially conflicting phylogenetic signals in this set of eleven nuclear markers. Overall, our data does not strongly discriminate between, or statistically reject, monophyly or paraphyly of the southern swordtails. Further studies are required to determine the evolutionary history of the southern swordtails, yet our RADseq markers and strongly support the monophyly of the southern swordtail clade and their basal placement (Figure 1.2b) in the genus.

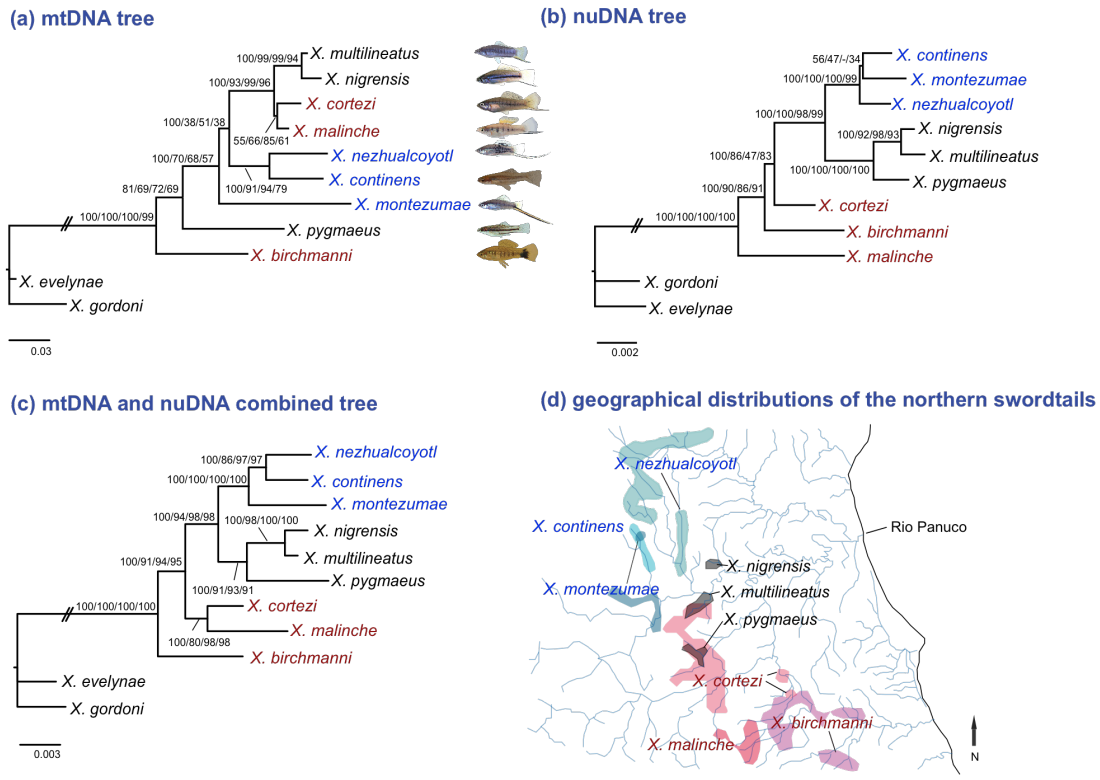

Figure 1.3 Mitochondrial and nuclear phylogenies of the nine northern swordtail species.

The phylogenetic trees were constructed from (a) combined sequences of two mtDNA loci (1235 bp) (complete control region and a segment of the cytochrome *b* gene), (b) combined sequences of eleven nuclear loci (7073 bp), and (c) combined sequences of two mitochondrial and eleven nuclear loci (8308 bp). Numbers indicate Bayesian posterior probabilities, Maximum-Likelihood, Neighbor-Joining and Maximum-Parsimony bootstrap values, respectively. The values of the branch length that was truncated are 0.115 (a), 0.007 (b) and 0.012 (c). Patterns of the geographical distributions of the nine species in the northern swordtails are shown (d) (map is modified from (Kallman & Kazianis 2006)). Species in the same clades inferred by (Rauchenberger *et al.* 1990) are shown in same color.

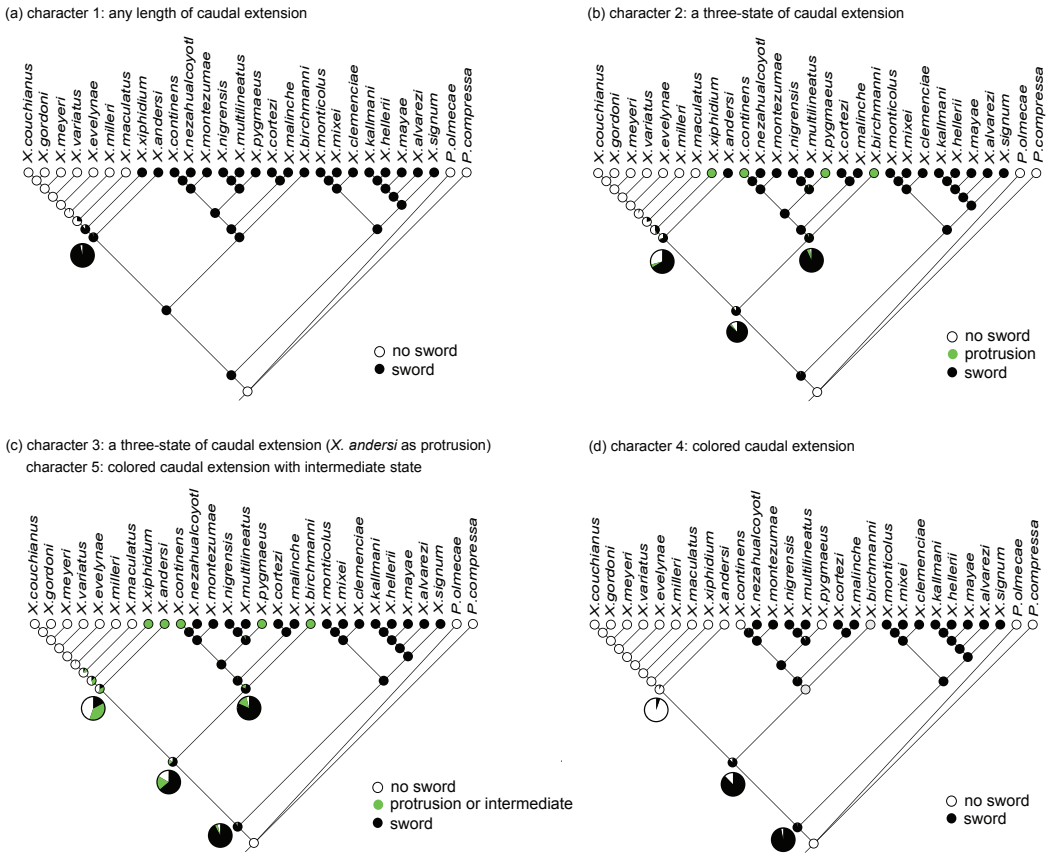

Figure 1.4 Maximum-likelihood reconstructions for the ancestral state of the sword in the genus *Xiphophorus*.

Five different characters were mapped onto the nuclear tree: (a) a two-state character of sword extension (character 1), (b) a three-state character of sword extension (no sword, protrusion and sword; *X. andersi* was coded as a sworded species, character 2), (c) a three-state character of sword extension (*X. andersi* was coded as a species with protrusion, character 3) (Meyer 1997) and colored caudal extension with intermediate state (character 5), and (d) a two-state colored caudal extension (*X. birchmanni* was coded unknown, character 4) (Wiens & Morris 1996). ML analyses of the character states, the colored caudal extension with polymorphic state (character 6), coloration (character 7) and the ventral black margin (character 8) of the sword, were not performed since Mesquite does not allow performing ML calculations on the characters that are polymorphic in some taxa. Each circle on the nodes represents character state (black filled circles: sword; green circles: protrusion or intermediate; empty circles: no sword; grey circles: unknown).

Ancestral state reconstructions of the sword

We constructed the ancestral state of the sword using our nuclear marker based phylogenetic tree that places the southern swordtail clade basal in the genus.

Several factors were considered in selecting a nuclear tree topology for the ancestral state construction of the sword. For the platies, we implemented a tree topology where *X. andersi* is basal to all platies, a topology also inferred by a previous study (Meyer *et al.* 2006). For the northern swordtail group, a tree topology based on the combined mtDNA and nuclear data sets was applied since it was always strongly supported at every node using different phylogenetic methods (Figure 1.3c). Monophyly of the southern swordtails was implemented (see above).

The definition of the sword and composite traits has long been debated and, on occasion differently interpreted in various species descriptions (Meyer & Schartl 1980; Rauchenberger *et al.* 1990; Basolo 1991; Meyer 1997). Also previous studies of ancestral state reconstruction have shown inconsistent ancestral states for the sword, depending on both how the sword was scored and which reconstruction methods were used (Wiens & Morris 1996; Meyer 1997; Schluter *et al.* 1997). Here, a comprehensive set of reconstructions of ancestral state was conducted by applying different sets of scoring of the sword (Figure 1.4 and Additional file 4) and by including other sets of sword traits (coloration, ventral black margin; Additional file 5), using both maximum likelihood (Figure 1.4 and Additional file 6) and parsimony approaches (Additional files 4, 5). Detailed descriptions of the sword characters and sword scorings are given in the Methods (see below).

In our maximum-likelihood analyses, the possession of a sword was always recovered as the ancestral state at the base of the genus *Xiphophorus* with high proportional-likelihood (0.921-0.996) in all character states (Figure 1.4, Additional file 6). However, in the parsimony analyses the ancestral states were not consistent since the ancestral state of the sword changed based on 1) how we treated the transition state of swords as ordered or unordered (i.e., protrusion is an intermediated step from ‘no sword’ to ‘sword’ states) and 2) how we defined the sword (i.e., only in terms of length or whether a sword also has to be a colored extension) (Additional file 4). The ancestral state of the sword was more ambiguous when the sword was coded as colored caudal extension (characters 4–6, see Additional file 4) rather than if only length of an extension of the caudal fin was considered to be a “sword” (characters 1–3, Additional file 4). However, clearly, none of the characters in any analysis

ever supported "swordless" as the ancestral condition in the genus *Xiphophorus*. Other sword traits (coloration, character 7; ventral black margin, character 8, Additional file 5) could not be reconstructed clearly because the absence or presence of these state of those traits was inferred to be equally parsimonious.

The sword clearly was lost at least once (more likely more than once, depending on the character state coding and reconstruction method) in this genus, but the repeated evolution of the sword could not be inferred strongly (Figure 1.4). It was lost once in the southern platy lineage when the sword was considered as a two-state character of sword extension (no sword, sword: character 1, Figure 1.4a). The sword appears to have "shortened" four times from a sword to short protrusion (or intermediate) during the evolution of this genus and it was inferred to have been lost completely once in the platy lineage under the definition of the sword as a three-state character of sword extension (no sword, protrusion or intermediate, and sword: characters 2, 3, 5, see Figures 1.4b, c). Another two-state character of the sword, the colored extension (character 4, Figure 1.4d), was lost twice in the northern swordtail lineage and once in the platyfish. These results suggest that the ancestral state at the root of the genus is "sworded". Furthermore, loss of sword or colored sword traits occurs much more frequently than their gain.

Discussion

Hybrid origin of *Xiphophorus monticolus*

We show that one of the recently described *Xiphophorus* species, *X. monticolus*, is likely to have arisen through an hybridization event, similar to what has been shown before for *X. clemenciae* (Meyer *et al.* 1994, 2006). The hybrid origins of the species, *X. monticolus* and *X. clemenciae*, are the most likely explanation for several reasons including, but not limited to their incongruous placement in the mtDNA and nuDNA trees (Figure 1.2). However, we cannot completely rule out that the discrepancy between phylogenetic placements based on different marker types does not result from other biological processes such as incomplete lineage sorting (ILS) (Sang & Zhong 2000; Degnan & Rosenberg 2009). However, we regard this as a much less plausible hypothesis. If ILS were the explanation for this discrepancy, a particular locus or group of loci would be expected to lead to different phylogenetic relationships from others. To evaluate whether particular loci have a particularly strong effect on the phylogenetic results, eleven separate nuclear trees were reconstructed by subtracting one locus at a time. Yet, we did not find evidence for strong locus-specific phylogenetic

relationships among all the eleven independent phylogenies analyzed with regard to the positions of the two hybrid species. Hybridizations among several species of *Xiphophorus* have been observed in the wild (Kallman & Kazianis 2006) and laboratory- hybrids can be produced for most species of *Xiphophorus* (Gordon 1947; Siciliano *et al*. 1971; Ozato & Wakamatsu 1981; Kazianis *et al.* 1996). All of these observations suggest that hybrid origins of species in this genus are feasible and appear to best explain the origin of these two species in this genus.

If *X. monticolus* and *X. clemenciae* indeed arose by hybridization it would be of great interest to know where and when this hybridization took place and which species are likely to be the parental lineages. We would like to point out that the sword indices (sword length / standard length) of *X. clemenciae* and *X. monticolus* are identical (0.25 for both species) whereas it is 0.06 for *X. mixei* (Kallman *et al.* 2004), the closest relative of both *X. monticolus* and *X. clemenciae* (Figure 1.2b) (Kallman *et al.* 2004). The high similarity in sword length and gonopodium (male modified anal fin for internal fertilization) structures particularly in ray 4 or 5 between *X. clemenciae* and *X. monticolus* might further suggest that both species arose from hybridization between a platyfish and the same paternal species. In the case of *X. clemenciae*, the discrepancy in the phylogenetic position was parsimoniously explained by an ancient hybridization event between a swordless platyfish, such as *X. maculatus* (or *X. milleri*), as the maternal species, and a sworded southern swordtail as the paternal species, such as *X. hellerii* in the previous studies (Meyer *et al.* 1994, 2006; Jones *et al*. 2012). Meyer *et al*. (Meyer *et al.* 2006) proposed that relatives of *X. maculatus* and *X. hellerii* might be parental species for *X. clemenciae* because those two species are closely related to *X. clemenciae* (in mtDNA and nuclear DNA, respectively) and have wide and overlapping geographic distributions. In addition, Meyer *et al*. (Meyer *et al.* 2006) found that laboratory crosses between female *X. maculatus* and male *X. hellerii* resulted in hybrids with intermediate sword lengths. They argued that because *X. clemenciae* has an intermediate sword length between *X. maculatus* (no sword) and *X. hellerii* (sword), *X. clemenciae* may have arisen from two species such as those.

Here we suggest that *X. monticolus* may also have originated from a hybridization event between a southern platy and a southern swordtail, and again species such as *X. maculatus* (or an ancestor of *X. maculatus*) may be the maternal species and *X. hellerii* or alternatively *X. mixei* (or an ancestor of those species) may be paternal species. Although other platies (e.g., *X. variatus*, *X. evelynae* and *X. milleri*) are genetically more closely related to *X. monticolus* (Figure 1.2a), as is the case of *X. clemenciae, X. maculatus* (or its ancestor)

is deemed to be the more likely maternal species because of its wider geographic distribution (see Figure 1.1). We caution that, of course, the current distributions may not necessarily resemble ancient distributions and therefore inferring parental lineages using current geographical information may not be reliable. Additionally, our comparative morphological study of *Xiphophorus* species revealed that *X. maculatus* has more similar gonopodial structures to the species in the *clemenciae* clade (e.g., *X. mixei*) than other platies (Jones *et al*., unpublished data). If the gonopodial lock-and-key hypothesis applies, i.e. that genital morphology may prevent mating between different species (Masly 2012), similar gonopodial structures between maternal (*X. maculatus*) and paternal species (*X. mixei*) might also lend further support to *X. maculatus* as a potential maternal species. Our favored hybridization scenario involves repeated backcrossing of hybrid females into the parental species with the longer sword which would explain that in terms of nuclear genes the hybrid species much more resemble their paternal species, as is clear shown in the nuclear DNA trees.

A *Xiphophorus mixei*-like fish is a likely alternative paternal lineage because it is the most closely related species to *X. monticolus* and *X. clemenciae* (Figure 1.2b) and the monophyly of *"clemenciae* clade" was strongly supported both phylogenetically and morphologically (Figure 1.2b, Kallman *et al.* 2004). The three species in the *clemenciae* clade (*X. clemenciae*, *X. mixei* and *X. monticolus*) all have orange lateral stripes that are produced by carotenoid pigments, whereas members of the *hellerii* clade exhibit red stripes where the pigment is produced by drosopterin (Kallman & Bao 1987). The spots (in red for *X. clemenciae* and in black for *X. mixei* and *X. monticolus*) in the proximal portion of the caudal fin in adult males were only detected in the *clemenciae* clade, but not in any other species in the genus *Xiphophorus* (Kallman *et al.* 2004). The species within each of the *clemenciae* and *hellerii* clades have more similar gonopodial structures to one another (Kallman *et al.* 2004), in particular, the distal part of the gonopodia, including hook shape, spine numbers and slightly anteriorly angled tip of the ramus is more similar between species in the *clemenciae* group in comparison to species in the *hellerii* group (Kallman *et al.* 2004). Furthermore, an ongoing comparative study of the gonopodium of the genus *Xiphophorus* further supports the hypothesis that the distal structures of *X. mixei* are similar to both species of hybrid origin in the *clemenciae* clade than any other species in the *hellerii* clade (Jones *et al*., unpublished data).

Xiphophorus monticolus may have arisen from a "local" hybridization event since it is restricted geographically to headwater streams of the Rio Jaltepec, a major Rio Coatzacoalcos tributary, in Oaxaca, Mexico (Kallman *et al.* 2004) (Figure 1.1b). Hence, *X. monticolus*,

similar to what we believe to be the case for *X. clemenciae* as well, is likely to have arisen from a hybridization event at a single locality. Thus, genetic similarity, shared morphological traits, and their sympatric distributions (Figure 1.1b) further support the hypothesis of a hybridization event between the species related to *X. maculatus* and *X. mixei*.

Combined all phylogenetic, phenotypic and distribution data point towards *X. mixei* as a likely paternal lineage for the two independent hybridization events within the *X. clemenciae* clade. Further genetic studies of *X. mixei* are warranted to uncover more genetic traces of this species that might support the hypothesis that it was involved in these hybridization/ speciation events. For example, distinct genetic mechanisms such as hemi-clonal lineages found in *Poeciliopsis monacha* may be analogous to the genesis of new hybrid species in the genus *Xiphophorus* (Mateos &Vrijenhoek 2002). *Poeciliopsis monacha* has an all-female system of reproduction, where females produce hybridogenic progeny that carry the maternal genome of *P. monacha* and replace it with a paternal nuclear genome of sexually reproducing species (e.g., *P. lucida*) in each generation (Mateos &Vrijenhoek 2002). Further research on ecological and behavioral aspects of *X. mixei* (e.g., female preference for the sword or other sexually selected traits) might provide interesting insights into the role of this species in the hybrid origin of its two closest relatives.

Phylogenetic relationships among the northern swordtails

Combined mitochondrial and nuclear data (Figure 1.3c) provide a more resolved phylogeny compared to previous phylogenetic analyses. However, these results should still be regarded as tentative. For instance, the monophyly and relationships within the *coterzi* clade, and their relationships to other lineages remain unresolved, as examined in several previous investigations (Borowsky *et al* 1995; Morris *et al*. 2001; Meyer *et al.* 2006; Gutierrez-Rodriguez *et al.* 2007, 2008).

Many incongruent relationships identified by the two types of marker-based phylogenies might provide a hint for another putative hybrid origin from the northern swordtail species. For example, the mitochondrial data set indicates a well-supported sistergroup relationship between *X. nezahualcoyotl* (long sword) and *X. continens* (protrusion), whereas the nuclear DNA set suggests that *X. nezahualcoyotl* could be closely related to *X. montezumae* (long sword). However, the nuclear tree could not provide strong support for the grouping of *X. nezahualcoyotl* with *X. montezumae*, so further analyses using additional nuclear markers are required to confirm this relationship. If this relationship is

supported by future analyses, as it actually is by our RADseq data (Jones *et al.* 2013), then it is likely that *X. nezahualcoyotl* might be of hybrid origin as well, on the basis of the incongruence of mitochondrial and nuclear trees. Further support for this hypothesis is provided by the notable morphological incongruence between the sister species *X. nezahualcoyotl* (long sword) and *X. continens* (short sword) in the mitochondrial tree (Figure 1.3a). This might suggests that hybrid speciation is not that uncommon within the genus *Xiphophorus* with hybrid origins for two of the 26 *Xiphophorus* species. Further studies are required to investigate the prevalence of hybrid origins and its overall role in speciation within the family Poeciliidae. However, several natural hybrid zones have been reported for some species-pairs (e.g., *X. birchmanni – X. malinche*) in the northern swordtail clade (Rosenthal *et al.* 2003; Gutierrez-Rodriguez *et al.* 2008; Culumber *et al.* 2011). Possibly some specimens in this group might have been collected from hybrid zones, although we lack detailed information about the sampling localities for some specimens of this study (Additional file 7). Therefore, phylogenetic placements of those species need to be interpreted with caution.

Evolutionary history of the sexually selected trait: the sword

The (repeated) loss of the most conspicuous aspects of the sword - its length and its coloration (Figure 1.4) - might suggest that the selective forces of natural selection repeatedly won over the forces of sexual selection that would tend to favor more obvious and exaggerated traits. The evolution of the sword clearly shows periods of reversals where a conspicuous long sword secondarily became less conspicuous and or shorter or was lost completely as at the origin of the platyfish. In future work it would be interested to investigate under what environmental conditions or biotic conditions natural selection might act most strongly against conspicuous and long swords. Several abiotic and biotic factors might play a role: (a) flow velocity of streams as longer sworded males would be expected to be at a hydrodynamic disadvantage, particularly in faster flowing streams, (b) water clarity and thereby visibility to females, but also predators, might also tend to select against males with particularly colorful or contrast swords (black strip), (c) particularly if the abundance of predators, in the water or from the air, that hunt visually or whose capture method might select against males with particularly long swords, that would tend to have a slower fast-start performance, is high.

The ancestral state of the sword remained disputed (Basolo 1991; Wiens & Morris 1996; Meyer 1997; Schluter *et al.* 1997). Previous character state reconstructions, based on the parsimony method (Meyer 1997), illustrated that the ancestral state reconstruction of the sword varied, depending on how the sword was categorized (i.e., no sword, protrusion and sword), and whether the transition between the two states (i.e., swordless and sworded) was treated as ordered or unordered. The use of likelihood reconstruction methods has similarly not provided a clear reconstruction of the ancestral state of the sword (Schluter *et al.* 1997). In the latter analysis the authors suggested two possible factors that might lead to uncertainty. One is that the "sword" was coded as a single trait with only two character states (i.e., no sword and sword) instead of three character states (i.e., no sword, protrusion, and sword). The other is possibly a higher rate of changes in sword character states in *Priapella* (a genus that has no sword at, just as all other poeciliids outside the genus *Xiphophorus*) than in *Xiphophorus* although the latter clade may have higher transition rate (Schluter *et al.* 1997). The ambiguity of the ancestral states in previous studies might also be affected by incomplete phylogenies since all previous analyses on the reconstruction of the evolution of the sword were conducted based on morphology-based phylogenies, where platies are basal to swordtails, or mtDNA-based trees where the four new species could not yet be included (Basolo 1991; Wiens & Morris 1996; Schluter *et al.* 1997; Meyer 1997). Mitochondrial phylogenies in particular were certainly misleading due to the incorrect placement of *X. clemenciae* among the platies as well as uncertainty about the topology of the tree in the deepest nodes and among the northern swordtails.

Our trait reconstructions using likelihood analyses covering all plausible sword scorings that have been used in several previous studies, implemented based on the nuclear marker based tree, show a consistent ancestral state, regardless of the different definitions of what constitutes the composite trait "sword". These results clearly indicate that the sword very likely originated in the common ancestral lineage of the entire genus *Xiphophorus*, and that the sword has been lost completely (no sword) or partially (protrusion or intermediate) in different lineages independently and repeatedly (see above) (Figure 1.4).

By comparison, the parsimony analyses still showed ambiguous ancestral states under the three character states (character 3, unordered; character 4; character 5, unordered) (see Additional file 4) although the same character state codings as in the likelihood method were applied and both sets of analyses used the identical comprehensive nuclear phylogeny. Of course, the maximum parsimony method reconstructs the ancestral state that requires the smallest number of state changes. It has been suggested that ecological traits are prone to

being biased because of the assumption of low rate of character changes or a stochastic element in parsimony analysis itself (Schluter *et al.* 1997). The ambiguous state reconstruction might therefore result from the method *per se* because changes in the characteristics of the sword are, apparently not that rare in *Xiphophorus*. Nevertheless, "swordless" was not found to be the ancestral state for the genus based on the parsimony analyses (Additional file 4).

Our analyses further suggest that the evolution of the sword is tending towards a reduction in length or even complete loss of the trait rather than secondary gains. Although the evolutionary origin of swords through female preference has been a central focus in the literature (Basolo 1990a, b, 1991, 1995a, b; Meyer *et al.* 1994, 2006), little attention has been paid to the 'loss' or 'reduction' of the sword or other characteristics of the sword such as its coloration or the black stripe. The brightly coloration of sword will increase the conspicuousness of its bearers not only to females, but also to predators and therefore be disadvantageous for survival (Rosenthal *et al.* 2001). Additionally, increased sword length is costly for fast start (escape) and endurance swimming (Basolo & Alcaraz 2003). If female preference is a major driver for the evolution of the sword, a change or loss of female preference might also equally affect the loss of the sword traits (i.e., length or coloration), or since female preferences for the sword seem to have been retained in species, such as platies, in which their males had lost their swords, natural rather than sexual selection might have played a decisive role in that (Meyer *et al.* 1994) (and see above). Alterations in female preference for the sword have been reported – for example, females prefer swordless conspecifics over sworded heterospecific males in *X. birchmanni* (Wong & Rosenthal 2006), and a change in female preference for the sword has been shown also for *X. nigrensis* (Rosenthal *et al.* 2002; Cummings *et al.* 2006).

If changes in female preference for the sword have indeed occurred frequently in response to different/changing environments or other factors impacting on mate choice, then our hypothesis that *X. mixei* constitutes the closest relative to the putative paternal lineage of *X. monticolus* (given that *X. mixei* has a relatively short sword) would gain further plausibility. This still needs to be tested in *X. mixei* females. It is also interesting to note that *X. mixei*, with its very short sword, is consistently nested within the *clemenciae* clade. So, despite being genetically closest to the hybrid species, and the putative "parental" lineage, *X. mixei*'s sword is very different from that of both *X. clemenciae* and *X. monticolus*. This observation, again, supports the notion that sword evolution can be fast and the phenotype is labile.

Future studies on the evolutionary relationships among *Xiphophorus* species would benefit from the inclusion of alternative outgroups since distant outgroups can possibly generate reconstruction artifacts. A robust phylogeny of the entire family Poeciliidae would help to clarify which species or genera are most closely related to the genus *Xiphophorus*. Further studies on ecological and behavioral features affecting the evolution of particular sword traits and the genetic compositions of hybrid species will help to unravel the origin of the repeated ancient hybridization events in the genus *Xiphophorus*. In a broader context, our study provides information on the long-standing controversy over the role and significance of hybridization in contributing to speciation and evolution in animals.

Conclusions

In this study, we reconstructed the phylogenetic relationships of all known 26 species in the genus *Xiphophorus* using eleven nuclear loci and two mitochondrial loci. We show that an additional (to the previously known *X. clemenciae*) southern swordtail species, *Xiphophorus monticolus,* is likely to have arisen through hybridization. Our mitochondrial and nuclear marker based phylogenetic analyses showed discordance in the position of this species, similar to previous analyses of *X. clemenciae.* The other three other recently described species - *X. mixei*, *X. kallmani* and *X. mayae* - group together with the southern swordtail species in both sets of trees. Among the northern swordtails, while the monophyly of the *montezumae* and *pygmaeus* clades was strongly supported in the nuclear phylogeny, our data also suggest incongruent phylogenetic relationships between two types of marker-based trees. The ancestral state reconstruction of the sword, in particular using maximum likelihood approaches, strongly suggests that the common ancestor of the genus *Xiphophorus* already possessed a sword and that it was lost completely secondarily again in the derived platy lineage of the genus. Our complete molecular phylogeny of the genus *Xiphophorus* provides a comprehensive phylogenetic framework within which the results of all studies on this genus can now be interpreted.

Methods

Taxon sampling

Seventy-three individuals from all described species of the genus *Xiphophorus* (26 species) including 16 specimens from the recently described four species (*X. kallmani*, *X. mayae*, *X.*

mixei and *X. monticolus*) were used. *Xiphophorus mixei* and *X. monticolus* were obtained from the *Xiphophorus* Genetic Stock Center (http://www.xiphophorus.txstate.edu, San Marcos, TX, USA). Two outgroup species, *Priapella compressa* and *P. olmecae* (Hrbek *et al.* 2007), were chosen based on our recently reconstructed a phylogeny for the poeciliids using several mitochondrial and nuclear DNA markers combined (7942 bp). The genera *Heterandria* and then *Priapella* were found to be the most closely related taxa to *Xiphophorus*, but *Heterandria* species showed a longer branch than *Priapella* species (Kang & Meyer, unpublished data). Sampling localities and detailed information about each of the samples are shown in Additional file 7 (lack of locality information is also indicated as 'unknown').

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from fin clips or tissues preserved in ethanol using a Qiagen DNeasy tissue kit (Qiagen). Ethanol preserved samples were incubated in TE9 buffer (500 mM Tris, 20 mM EDTA, 10 mM NaCl, pH 9.0) overnight to improve the extraction efficiency by eliminating an excess of ethanol (Chakraborty *et al.* 2006). Polymerase chain reaction (PCR) was performed in 15 μl volumes containing 1x PCR buffer, 200 μM each dNTP, 1.5 mM $MgCl_2$, 0.6 μM each primer and 1U of High fidelity *Taq* polymerase (Fermentas).

We used published primers of the mitochondrial control region, cytochrome *b*, Rag1/exon 3, X-*src* and three non-coding flanking regions of the microsatellite loci (D2, D8 and T36) to obtain the sequences for the four newly described species. Sequences of these seven loci for the 22 remaining species were acquired from GenBank (see below). Six primer-pairs were newly designed to amplify the intronic regions of nuclear loci: GNG 13, G6PD (6^{th} intron), UNG (4^{th} intron), POLB (7^{th} to 11^{th} intron), FEN1 (3^{rd} intron) and TP53 (4^{th} intron) for all 26 species (See Table 1.1). For the GNG 13 locus, degenerate primers were designed based on the conserved exon flanking regions from five teleost species (zebrafish, medaka, fugu, tetraodon and stickleback) and the other five primer-pairs were developed using *X. maculatus* genomic sequences (which were provided by Ron Walter, *Xiphophorus* Genetic Stock Center, Texas State University, USA). DNA sequences of these new six primer-pairs and PCR conditions are provided in Additional file 8.

PCR was carried out using appropriate conditions for each primer pair, for example, 94°C for 3 min, 35 cycles at 94°C for 30 sec, 42-60°C for 30 sec and 72°C for 1-1min 30 sec.

A final extension step at 72 °C (7 min) was conducted. PCR products were checked on 1.5 % agarose gels, and then incubated at 37°C for 15 min and 85°C for 15 min with Exonuclease I and Shrimp Alkaline phosphatase (Fermentas), respectively in order to purify the PCR products (Werle *et al.* 1994). The purified mtDNA and nuclear DNA fragments were subject to direct sequencing in the forward and reverse directions using the same forward and reverse primers as in the PCR and the BigDye Terminator 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). All DNA sequencing reactions were run on a 3130*xl* DNA Analyzer (Applied Biosystems) and analyzed with ABI PRISM DNA Sequencing Analysis Software version 5.3.1. Both forward and reverse strands were sequenced for accuracy in each individual.

Phylogenetic analyses

DNA sequences were analyzed from all 26 species plus two outgroup species *Priapella compressa* and *P. olmecae*. Two combined mitochondrial gene sequences (1239 bp) and eleven combined nuclear gene sequences (7276 bp) were used separately in the phylogenetic analyses. In addition, a combined mitochondrial and nuclear phylogeny (8515 bp) was reconstructed.

For the two mitochondrial loci, sequences of cytochrome *b* (360 bp) and control region (879 bp) were acquired for the four new species and were combined with published sequences for the 22 remaining species plus two outgroup species deposited in GenBank (Meyer *et al.* 1994, 1996). The total length of sequences, the number of variable sites, parsimony informative sites, and the nucleotide diversity of each mitochondrial and nuclear locus are shown in Table 1.1.

For the eleven nuclear loci, previously published sequence data from 22 species and the two outgroup species were used for five nuclear loci (Rag1/exon 3: 1574 bp, X-*src*: 520 bp, D2: 393 bp, D8: 516 bp and T36: 394 bp) (Meyer *et al.* 1994, 1996), and new sequence data for the four newly described species were determined for these loci. Our newly developed six intron-makers were amplified for all 26 species and the two outgroup species. The sequences of the intronic regions of the genes, GNG13 (531 bp), G6PD (526 bp), UNG (277 bp), POLB (672 bp), FEN1 (827 bp) and TP53 (1046 bp) were combined with the previously published sequences of the other five loci in an effort to construct a nuclear marker phylogeny.

In addition to the phylogenetic analysis of all the 26 *Xiphophorus* species, phylogenetic relationships solely for the northern swordtail clade consisting of nine species (*X. birchmanni*, *X. continens*, *X. cortezi*, *X. malinche*, *X. nezahualcoyotl*, *X. montezumae*, *X. nigrensis* and *X. pygmaeus*) with two outgroups, *X. gordoni* and *X. evelynae* (northern and southern platies that are sister taxa) were reconstructed. This analysis was performed in an effort to resolve the phylogenetic relationships within this group only because the phylogenetic relationships of the northern swordtails were weakly supported and conflicts on the tree topologies were found among different phylogenetic inferences in the whole species tree (Figure 1.2). The same sequence matrix was used except that columns with gaps only caused by the other species were deleted. Therefore, a total length of 1235 bp (mitochondrial phylogeny, Figure 3a) and 7073 bp (nuclear phylogeny, Figure 1.3b) sequences was used for these phylogenetic analyses of the northern swordtail group. We also reconstructed a phylogenetic tree using a combined mitochondrial and nuclear data set (8308 bp) (Figure 1.3c).

The sequence alignment was carried out using the Clustal-W multiple sequences alignment package (Thompson *et al.* 1994) implemented in BioEdit 7.0 (Hall 1999) that was then manually adjusted by eye (Additional file 9). We conducted Bayesian Inference (BI), Maximum-Likelihood (ML), Neighbor-Joining (NJ), and Maximum Parsimony (MP) analyses for the phylogenetic reconstruction. These analyses were performed separately for the two combined data sets of two mitochondrial and eleven nuclear genes. The MP and NJ analyses were conducted using MEGA 4.0 (Tamura *et al.* 2007). Bootstrap probabilities were obtained with 1000 replicates (Felsenstein 1985). For ML and BI analyses, best models of nucleotide substitutions were tested separately for mtDNA and nuclear DNA data sets using jModeltest 0.1.1 (Posada 2008) under the Akaike Information Criterion (AICc) using a corrected version for small samples (Sugiura 1978). In cases where the models selected by jModeltest were not available in the phylogenetic construction program, the next fit model was applied (Table 1.1).

ML analyses were performed using PhyML 3.0 (Guindon *et al.* 2010) and statistical support was obtained with 1000 bootstrap replicates. A Bayesian Markov Chain Monte Carlo approach (Yang & Rannala 1997; Mau *et al.* 1999) was used as implemented in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Three partition schemes including 1) each locus separated, 2) coding and noncoding, and 3) no partition were tested in PartitionFinder 1.0. (Lanfear *et al.* 2012). The partition for each locus separated was selected as the best scheme under AICc criterion. Two chains were run for 10,000,000

generations, starting from random trees that were sampled every ten generations yielding 10,000,000 trees and the first 25 % of trees were discarded as burn-in. An average standard deviation of split frequencies for both runs was less than 0.01, suggesting that concurrent runs converged. Potential scale reduction factor (PSRF) of 1.0 was found, which verifies that we have reliable samples from the posterior probability distribution.

The confidence of phylogenetic tree selection was accessed by the Approximately Unbiased (AU) and Shimodaira-Hasegawa (SH) tests implemented in CONSEL (Shimodaira & Hasegawa 2001). To do so, the log-likelihoods of site-patterns of the best ML unconstrained trees (Figure 1.2) were estimated using Tree-Puzzle (Schmidt *et al.* 2002) under the 'user defined trees' mode, assuming GTR+G model for nuclear DNA phylogeny and HKY+G+I model for mtDNA phylogeny. Then both the mitochondrial and nuclear best ML constrained trees regarding the positions of *X. monticolus* and *X. clemenciae* were reconstructed using RAxML with constraint option (see the result) (Stamatakis *et al.* 2008). Finally, the best ML unconstrained and constrained trees were compared with each data set in CONSEL (Shimodaira & Hasegawa 2001). The confidence for each tree topology was presented as *p*-value (Table 1.2). Additionally, exhaustive search of the ML tree for the northern swordtail group was performed. A strongly supported node (100 of bootstrap value) consisting of three species (*X. multilineatus*, *X. nigrensis* and *X. pygmaeus*) was constrained to reduce the number of OTUs due to computational constraints.

Ancestral reconstructions of the sword

Ancestral states of swords were reconstructed under parsimony and maximum-likelihood approaches implemented in Mesquite 2.72 (Maddison & Maddison 2010). The swords are a composite character including the extension of the caudal fin, coloration of the sword and black pigmentation of the ventral margin (Basolo 1990a, 1995a). Here, we used sword characters as defined in Meyer (Meyer 1997) (characters 1–3), Wiens and Morris (Wiens & Morris 1996) (character 4) to include more characters of the sword than only the length of sword. Detailed descriptions of our sword scorings are as follows.

In our maximum-likelihood reconstruction, for character 1 (a two-state) any length of caudal extension was considered as a sword (Meyer 1997). Characters 2 and 3 were scored as a three-state character (no sword-protrusion-sword) (Meyer 1997). Character 2 coded *X. andersi* as sworded, but as a protrusion in character 3. Character 4 adopted colored elongation as a sword (Wiens & Morris 1996). Further, we added one more character to

consider the polymorphic state of the colored protrusion (character 5). This was applied for a more comprehensive sword scoring based on previous studies (Wiens & Morris 1996; Meyer 1997) and our field observation in nature. For character 5 a colored extension was considered as a sword, and species showing a short sword (i.e. protrusion), colored protrusion, or polymorphism for a protrusion or coloration were assigned to the "intermediate" category. However, this scoring is the same as described for character 3 (i.e. intermediate instead of protrusion) so here we do not show the reconstruction result for character 5 separately (see character 3) (Figure 1.4c).

In our parsimony approach, we used the same scorings as for characters 1–5 as in the maximum likelihood method (Additional file 4). We also added one more character (character 6) in order to apply the polymorphic state of the colored caudal extension since such polymorphic states can only be used in parsimony analyses in Mesquite (Maddison & Maddison 2010). Three northern swordtails (*X. birchmanni, X. continens*, and *X. pygmaeus*) and *X. xiphidium* were also coded as polymorphic for the colored caudal extension according to Meyer (1997) and our field observations (character 6, Additional file 4). In addition to the ancestral state reconstruction of the sword itself, the ancestral state of other sword traits (coloration: character 7 or ventral black margin: character 8) were also reconstructed separately (Additional file 5) (Meyer 1997). Coloration of the sword in *X. continens* was also coded as polymorphic because sub-dominant males have an unpigmented protrusion in the wild (character 7; Additional file 5).

The evolutionary history of each of character states was reconstructed based on what would be currently the best phylogenetic hypothesis for this genus obtained by maximum-likelihood inference. The character states were treated as "ordered" and "unordered" in the parsimony reconstructions. The unordered transition state permits the transformation of a character state to any other state. The ML reconstruction was performed with the MK1 (Markov k-state 1 parameter model) model of evolution, which is a k-state generalization of the Jukes-Cantor model and under this model any particular character state changes have equal probability (Pagel 1999; Lewis 2001). Mesquite does not allow performing ML calculation on the characters that have polymorphism for some taxa. Therefore, only parsimony analyses were performed on the character 6, 7 and 8 (Additional files 4, 5).

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Chapter 2. Retinoic acid is involved in the metamorphosis of the anal fin into an intromittent organ, the gonopodium, in the green swordtail (*Xiphophorus hellerii*)

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Retinoic acid is involved in the metamorphosis of the anal fin into an intromittent organ, the gonopodium, in the green swordtail (*Xiphophorus hellerii*)

Nils Offen*[1, 2], Ji Hyoun Kang*[1,3], Axel Meyer[1, 3], Gerrit Begemann[1, 4, §]

[1] Lehrstuhl für Zoologie und Evolutionsbiologie, Department of Biology, University of Konstanz, Konstanz Germany

[2] Lund Strategic Research Center for Stem Cell Biology and Cell Therapy, Dept. of Laboratory Medicine, Lund University, Lund, Sweden

[3] Konstanz Research School Chemical Biology, University of Konstanz, Konstanz, Germany

[4]Developmental Biology, University of Bayreuth, Bayreuth, Germany

* These authors contributed equally to this work

[§]Corresponding author:
Gerrit Begemann
Developmental Biology
University of Bayreuth
Universitätsstraße 30
95440 Bayreuth, Germany
Tel: +49-(0)921-55-2475
Fax:+49-(0)921-55-2794
E-Mail: gerrit.begemann@uni-bayreuth.de

Abstract

In poeciliid fish the male anal fin has been transformed into a gonopodium, an intromittent organ required for internal fertilization. Elevated testosterone levels induce metamorphosis of a subset of anal fin rays to grow and form the specialized terminal structures of the gonopodium. The molecular mechanisms underlying these processes are largely unknown. Here, we investigated whether retinoic acid (RA) signaling is involved in gonopodium development in the swordtail *Xiphophorus hellerii*. We showed that *aldh1a2*, a RA synthesizing enzyme, and the RA receptors, *rar-ga* and *rar-gb,* are expressed in anal fins during metamorphosis. *aldh1a2* expression is regulated by testosterone in a concentration-dependent manner and is up-regulated in both hormone-induced and naturally developing gonopodia. *Androgen receptor* (*ar*), a putative regulator of gonopodial development, is co-expressed with *aldh1a2* and the RA receptors in gonopodial rays. Importantly, experimental increase of RA signaling promoted growth of the gonopodium and increased the number of new segments. Based on gene expression analyses and pharmacological manipulation of gonopodium development, we show that the RA signaling pathway is activated in response to androgen signaling and promotes fin ray growth and development during the metamorphosis of the anal fin into the gonopodium.

Introduction

A majority of all extant fish species (> 95 %) belongs to the group of ray finned fish. Given more than 23,000 species the diversity of this group represents approximately 50 % of all living vertebrates. Most of these fishes (98 %) exhibit an oviparous mode of reproduction, while in at least 54 fish families 'viviparous' reproduction exists (Wourms 1981). One of those families are the Poeciliid fish (Family: Poeciliidae), which consist of the three subfamilies, Aplocheilichthyinae, Procatopodinae and Poeciliinae (Ghedotti 2000). The Poeciliinae is one of the three groups within the toothed carps (suborder Cyprinodontoidei) that are thought to have independently evolved internal fertilization and a specialized intromittent organ (Meyer & Lydeard 1993). The intromittent organ found in poeciliid fish, called 'gonopodium' is sexually dimorphic and develops from the anal fin rays 3-5, the so-called 3-4-5 complex, during sexual maturation (Langer 1913; Parenti 1981). These rays are a modified structure in terms of ray length, segment thickness and distal structures like blades, claws, spines, hooks and serrae (Gordon & Rosen 1951). The morphological characteristics of the gonopodium, particularly its terminal structures, greatly vary across species and thus have extensively been used for phylogenetic analysis (Rosen & Bailey 1963). By comparing the three gonopodial rays and their distal structures among several species (Gordon & Rosen 1951), Gordon and Rosen (1951) studied the species-specific variability in the gonopodium morphology of *Xiphophorus* species including the green swordtail (*X. hellerii*) (see Figure 2.1A). While ray 3 is not bifurcated, rays 4 and 5 bifurcate into two sister rays, an anterior (a) and a posterior (p) parts (Figure 2.1B). Spines or serrae are formed by the rays 3 and 4p and the number of those is quite variable at the inter- and even intra-specific levels. In addition, ray 3 exhibits a terminal hook. Ray 5a carries a terminal claw that is usually present, but completely lost in some species. Furthermore, a terminal blade develops between the rays 3 and 4a in a species-specific manner in the genus *Xiphophorus*. The growth and segmentation rates can differ between the gonopodial rays, which results in segments of different lengths during the gonopodium development (Rosen & Bailey 1963). In general, the final length of the gonopodium depends on the body size of the individual fish (Rosen & Bailey 1963).

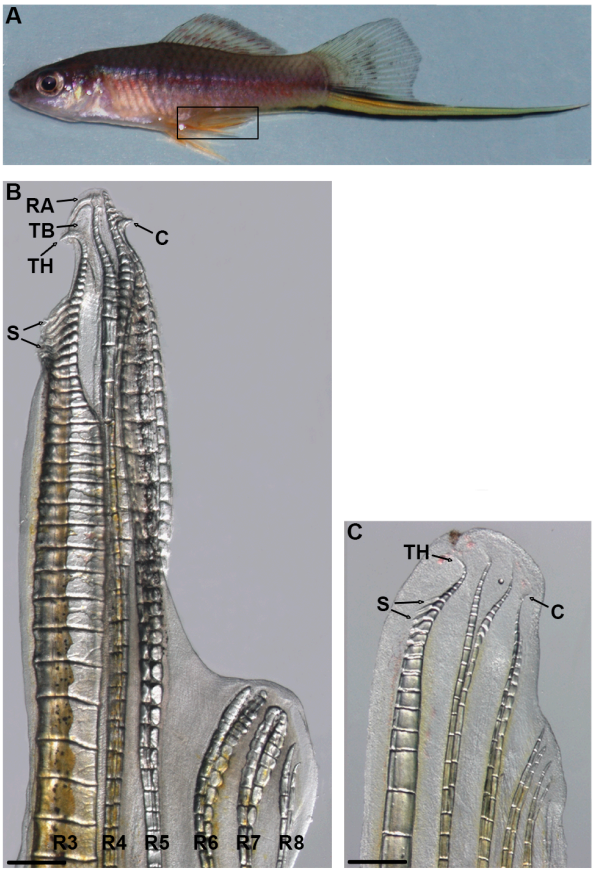

Figure 2.1 Structure of the male gonopodium.

In adult swordtail males the anal fin forms a gonopodium (bracket, A). A naturally developed gonopodium with typical terminal structures that form distal lepidotrich segments (B) and a testosterone-induced gonopodium (24 days of testosterone treatment) (C); caudal is to the top and ventral to the left (B, C). Arrows indicate terminal structures. Abbreviations: C, claw; RA, ramus; TB, terminal blade; S, spine; TH, terminal hook. Scale bars: 500 µm.

Metamorphosis in male swordtails (genus *Xiphophorus*) transforms not only the anal fin into a gonopodium, but also the caudal fin into a "sword", which is modified from ventral caudal ray 7 to 10 (Dzwillo 1964). It has been suggested that the genetic network underlying the development of the sword is partly co-opted from the evolutionarily older gonopodium (Zauner *et al.* 2003). The sword evolved and was lost again secondarily in the lineage of the genus *Xiphophorus* (Jones *et al.* 2013; Kang *et al.* 2013) whereas the 3-4-5 complex of the gonopodium evolved once in the common ancestor of the poeciliid fish. The genetic network that regulates gonopodium development remains poorly understood. Androgen signaling has

been suggested to play a role in gonopodium development in *Gambusia affinis* and *X. maculatus*, since exogenous testosterone can induce gonopodium formation in juvenile fish (Turner 1941a; Grobstein 1942). In 1941, Turner postulated a two-phase model for gonopodium development (Turner 1941a, 1941b) : first, when the testis start to mature, low levels of testosterone are released and promote outgrowth of the gonopodial rays to form the 3-4-5 complex. As the amount of hormone released increases, the differentiation of the terminal structure occurs at the growing gonopodium in a site-specific manner and in a typical temporal sequence (Grobstein 1948). Gonopodial development can be induced in juvenile fish by the application of exogenous testosterone; however, there are differences between naturally developing and artificially induced gonopodia (Grobstein 1947; Turner 1947a; Sangster 1948). Induced gonopodia basically form all terminal structures, yet they are shorter and less differentiated (Figure 2.1C). It would be likely that high levels of testosterone induce fin ray growth followed by the terminal modification of fin rays at the same time (Turner 1941a; Grobstein 1942).

Independent support for the role of androgen signaling in gonopodium development is based on a study by Ogino and colleagues (Ogino *et al.* 2004). They found that both *androgen receptor a* and *b* are expressed in the developing gonopodium of *Gambusia affinis* and inhibition of AR signaling with flutamide perturbs gonopodium development. In addition, they identified *shh* as a target gene that is regulated by androgen signaling. *shh* and its receptor *ptc1* are expressed during gonopodium development of *G. affinis* and inhibition of *shh* signaling also blocks gonopodium development (Ogino *et al.* 2004). Moreover, *fgfr1* and *msxC* are up-regulated in growing gonopodial rays and are thought to promote gonopodium outgrowth (Zauner *et al.* 2003; Offen *et al.* 2008). Experiments by Pickford and Atz (1957) suggest a putative role of thyroid signaling. Treatments of juvenile fish with thyroid hormone resulted in anal fin ray growth (Pickford & Atz 1957). Environmental estrogenic biochemical can also induce gene expression of the estrogen receptor in the anal fin in medaka (Pickford & Atz 1957; Hayashi *et al.* 2007).

Retinoic acid (RA), a small lipophilic and diffusible chemical, is an important signaling molecule for embryonic development because it is known to be involved in many key developmental processes, such as somitogenesis, left-right asymmetry formation, heart development and neurogenesis (Maden & Corcoran 1996; Chazaud *et al.* 1999; Duester 2007; Rhinn & Dolle 2012; Shimozono *et al.* 2013). It is synthesised by a group of retinaldehyde dehydrogenases (Aldh1as) and stimulates gene expression through their

binding to two types of receptors, retinoic acid receptors (RARs) and retinoic X receptors (RXRs) (Morriss-Kay & Ward 1999). RA also plays a crucial role in the formation of paired appendages since it is essential for forelimb bud initiation in either mouse or zebrafish (Akimenko & Ekker 1995; Begemann *et al.* 2001; Grandel *et al.* 2002; Mic *et al.* 2004; Gibert *et al.* 2006; Grandel & Brand 2011). Furthermore, it is involved in proximo-distal patterning of skeletal elements in later stages of limb development (Mercader *et al.* 1999; Mercader *et al.* 2000; Mic *et al.* 2004). Furthermore, the morphogenic effect of RA signaling (i.e. patterning formation) was found in several fin regeneration studies (Geraudie *et al.* 1993, 1995; White *et al.* 1994; Laforest *et al.* 1998). Recently it was also shown that RA signaling regulates the blastema formation, proliferation and the survival of mature blastema during zebrafish fin regeneration (Blum & Begemann 2012).

Given RA signaling is required for the development of paired fins and fin regeneration, we explore the potential role of RA in the metamorphosis of an unpaired anal fin into the gonopodium in a green swordtail fish, *Xiphophorus hellerii*. We cloned *aldh1a2* and two *rarg* receptors and examined their expression patterns during the gonopodium development. The levels of gene expression of *aldh1a2* were quantified in both testosterone-induced and naturally growing gonopodia. Through manipulative experiments of over-activating RA signaling, we further investigate a role of RA in outgrowth of ray and addition of new lepidotrich segments of developing gonopodia. We show that during anal fin metamorphosis of gonopodium outgrowth *aldh1a2* is co-expressed with *androgen receptors* and *aldh1a2* gene expression increases in a testosterone concentration-dependent manner, suggesting that RA synthesis might be controlled by androgen signaling.

Materials and Methods

Ethics Statement

All experiments involving animals were performed in accordance with the German Animal Welfare Act and were approved by the Government of Baden-Württemberg, Regierungspräsidium Karlsruhe, Germany (G-09/105, G-10/122).

Fish stocks and maintenance

Xiphophorus hellerii were taken from stocks kept at the University of Konstanz. Fish were maintained on a 12:12h light:dark cycle at 24°C in 110-litre densely planted aquaria and were fed TetraMin flakes and Artemia.

***Cloning* aldh1a2, rar-ga, rar-gb *and* androgen receptors**

cDNA fragments of *raldh2*, *rar-ga* and *rar-gb* were isolated from recombinant phage DNA, derived from the *X. hellerii* λ-phage cDNA library (Offen *et al.* 2009), by PCR using degenerate Primers. A 767 bp *aldh1a2* fragment was amplified by PCR using the Primers raldh2-fw1: 5'-GGI TAY GCI GAY AAR ATH CAY GG-3'and raldh2-rev1: 5'-ACR TTI GAR AAI ACI GTI GGY TC-3'. A 602 bp *rar-ga* and a 603 bp *rar-gb* fragment were amplified by PCR using the Primers RAR-fw2: 5'-TGY GAR GGI TGY AAR GGI TT-3' and RAR-rev2: 5'-GGI CCR AAI CCI GCR TTR TG-3'.

To obtain appropriate size *rar-ga*/*rar-gb* fragments for RNA probe generation, the 3' ends of the cDNAs were amplified from the cDNA library using PCR with the primer pairs RAR1-fw1: 5'-GGA GAG CTT GAA GAA CTG GTC-3'/ lib-univ: 5'-CAC TAT AGG GCG AAT TGG CTA CCG-3' for *rar-ga* and RAR2-fw1: 5'-GAA CTG GAG GAG CTT GTG AAC-3'/ lib-univ for *rar-gb*. For *rar-ga*, a ~1,3 kb and for *rar-gb* a ~1,5 kb fragment was amplified. The PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the pCRII-TOPO vector (Invitrogen, Karlsruhe, Germany) for sequencing.

To obtain a fragment of both *androgen receptors* to generate an RNA probe giving a reliable signal, the phage λ-phage cDNA library was screened with DIG labelled RNA probe derived from *X. hellerii arb* and *arb* cDNA fragments (Offen *et al.* 2009). 10^6 recombinant phages were grown, transferred to nitrocellulose membranes (Nitropure 45 µm, Osmonics, Minnetonka, USA) and prepared for screening according to the ZAP-cDNA® Library Construction Kit manual (Stratagene, Heidelberg, Germany). The membranes were treated with Proteinase K (2 mg/ml) in PBS for 30 min at 37 °C, washed with ddH_20 and prehybridised in hybridisation buffer (50% Formamide, 5x Denhardt solution, 5x SSC, 0.1% SSC, 250 µg/ml sheared herring sperm DNA) for 1 h at 50°C. RNA probe was added and allowed to hybridise to the complementary cDNA for more than 16h at 50°C. Afterwards, membranes were washed five times for 10 min in 2x SSC with 0.1% SDS, two times at RT and three times at 42°C. After blocking unreacted binding sites on the membrane with 1% blocking agent (Roche, Mannheim, Germany) in maleic acid buffer (100 mM maleic acid,

150 mM NaCl) for 1 h, immunolabelling of hybridised probe was performed using a alkaline phosphatase coupled DIG antibody (1:2000 in maleic acid buffer; Roche, Mannheim, Germany) for 2h. After washing several times in maleic acid buffer, the antibody detection was performed as described for in situ hybridisation (Poss *et al.* 2000). The pBluescript phagemid containing the cDNA insert was excised from the λ -phage genome as described in the ZAP-cDNA® Library Construction Kit manual (Stratagene, Heidelberg, Germany).

RNA probe synthesis and whole-mount in situ hybridisation

For *in situ* hybridisation juvenile fish were either treated with 5 µg/l 17-α-methyltestosterone for a variable number of days. The testosterone or mock treatment was repeated every fourth day. At the end of the treatment fish were anesthetized and 2/3 of the anal and 1/3 of the caudal fin were amputated using a sterile razor blade. The fins were fixed in 4% paraformaldehyde in PBS overnight, transferred to methanol and stored at -20°C until use.

Antisense and sense RNA probes were generated using a digoxigenin labelling kit (Roche, Mannheim, Germany). Probes for *aldh1a2*, *rar-ga* and *rar-gb*, *ara* and *arb* were generated from the cDNA fragments listed above. *In situ* hybridisation on *Xiphophorus* fins were performed as described (Poss *et al.* 2000) with several modifications. Prehybridisation was done 4h at 68°C in formamide solution (50% formamide, 5x SSC, 0.1% Tween20, pH to 6 with 1 M citric acid). Post-hybridisation washing steps were initiated at 68°C with formamide solution. To block unspecific binding sites 0.5% blocking reagent (Roche, Mannheim, Germany) in PBT was used. Antibody incubation was done at 4°C overnight. After fixation of stained fins/blastemata, the tissue was washed twice 20 min in PBT, 20 min in ethanol/PBT (70:30) and 20 min in 100% ethanol and stored at 4°C. The specificity of anti-sense probes was verified with sense probe experiments.

***In situ* hybridisation on longitudinal sections**

Anal fins from individuals treated with 17-α-methyltestosterone for 7 days were fixed in 4% Paraformaldehyde (Sigma-Aldrich, Munich, Germany). Longitudinal sections of 10µm thickness were created using a Reichert-Jung Autocut 2040 Microtome and *in situ* hybridisation was performed as described (Kuraku *et al.* 2005).

Intraperitoneal (IP) injection of dissolved RA

Up to six juvenile individuals of *X. hellerii*, aged between 3 and 6 months, were placed in a 30-litre tank and treated as follows: Both RA injected and control groups were treated with 5 µg/l of 17-α-methyltestosterone to induce gonopodium development (1 mg/ml stock solution in ethanol; Sigma-Aldrich, Munich, Germany). Induction of gonopodia is achieved with 10 µg/l testosterone (Offen *et al.* 2009), but, as determined in this study, the maximum response is already obtained at 5 µg/l. 20 µl of 1 mM RA (all-trans retinoic acid in ethanol; Sigma-Aldrich, Germany), dissolved in phosphate buffered saline (PBS; Sigma-Aldrich, Munich, Germany), was injected into the peritoneum and the same volume of PBS was injected in animals of the control fish. The concentration for RA is an empirical value derived and adjusted for weight from routine injections in zebrafish (Blum & Begemann 2012). Testosterone treatment (day 0 and 4) and RA injection (day 2 and 6) were repeated. For analysis of morphological changes in early gonopodium development fish were anaesthetized by incubation in a solution of 0.08 mg/ml tricaine (3-aminobenzoicacid-ethylester-methanesulfonate; Sigma-Aldrich, Munich, Germany) and anal fins/gonopodia were photographed. Photographs were taken before (at day 0) and after (at day 7) the treatment using the AxioVision software v3.1 (Zeiss) and the digital camera Zeiss AxioCam MRc. The length was measured using the software ImageJ (Abramoff *et al.* 2004). The dataset was checked for normal distribution using graphical methods (normality plot) and statistical tests (Shapiro-Wilk). A t-test or Mann-Whitney test were used to test significant difference between RA injected and control group.

Quantitative real-time PCR (qPCR) in testosterone-induced and naturally developing gonopodia

Juvenile individuals of *X. hellerii* (n = 3) were treated with 17-α-methyltestosterone to a final concentration of 5 µg/l under the conditions described above. Anal fins (1/2) were amputated with a sterile razor blade at 7 days of treatment. Total RNA of anal fin tissue was isolated with Trizol reagent (Invitrogen, Karlsruhe, Germany). Genomic DNA contamination was removed by incubating total RNA with DNaseI (Fermentas, St. Leon-Rot, Germany). Reverse transcription was performed for each sample in a final volume of 20 µl with 200 ng of total RNA using the Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). Quantitative real-time PCR (qPCR) was performed with 0.4 ul of cDNA product with iQ™ SYBR® Green Supermix (Bio-Rad, Munich, Germany) using a C1000 thermal

cycler combined with a CFX96 real-time PCR detection system (Bio-Rad, Munich, Germany). The gene specific primers used are shown in Table 2.1. qPCR were performed according to the following program: 95 ºC for 15 sec, followed by 45 cycles of 95 ºC for 15 sec, 61.3 ºC for 30 sec and 72 ºC for 30 sec, then 95 ºC for 10 sec as a final step. The fold-change in expression of *aldh1a2* was analyzed using the ΔΔCt method, with β-actin as internal control (Livak & Schmittgen 2001).

Quantitative real-time PCR (qPCR) was also performed for naturally developing gonopodia. Males with a naturally developing gonopodium and females were obtained from a community tank. We classified into two different categories of males based on the developmental phase of gonopodium. "Developing" gonopodia (n = 4) was defined as thickened and extended ray and the number of segments at ray 3 were between 10 and 34. "Almost-mature" gonopodia (n = 8) was defined when differentiated distal structures were present, ray 3 had developed more than 35 segments, and a colored sword was visible in the caudal fin. Females (n = 5) were used as a control group; the number of ray 3 segments was 8 or 9 in all individuals.

The tip of the gonopodium (4 mm) was cut from each individual used for total RNA isolation. RNA preparation, synthesis of cDNA and qPCR were performed as described above. One-way ANOVA was followed by post hoc testing to test significant difference of the level of *aldh1a2* gene expression among different concentrations. Linear regression was performed to predict the effect of testosterone on gene expression levels. Delta Ct was used as dependent variable. A p-value lower than 0.05 was considered to be significant.

Table 2.1 Gene specific primers used for qPCR

Primer Name	Sequence (5'-3')	F or R	Locus	Amplicon size
Bact_337F	CAGTGGTTGGCGCATACTTA	F	b-actin 3'UTR-contig	208
Bact_544R	CCCCATGTTACCGTCACTTT	R	b-actin 3'UTR-contig	
AldhI_100F	GCCTCTCCACCCACATTAAC	F	*Aldh1a2*	234
AldhI_333R	GACCGAGTCTCTGAGCATCC	R	*Aldh1a2*	

Phylogenetic analysis and protein domains

cDNA sequences of retinoic acid receptors, *aldh1a* enzymes and androgen receptors were sampled from GenBank and Ensembl using the Blast algorithm (Altschul *et al.* 1990) and aligned using ClustalW (Thompson *et al.* 1994). For aldh1as the full cDNA alignment (excluding the third position) was used for the phylogenetic analysis. For the retinoic acid

and androgen receptors a cDNA fragment coding for the C4 zinc finger and the hormone binding domain was used to build the tree. Based on the alignments, phylogenetic relationships were constructed using maximum likelihood (ML) and Bayesian methods of phylogeny inference (Larget & Simon 1999). ML analyses were performed using PHYML 2.4 (Guindon & Gascuel 2003). The best fitting models of sequence evolution for ML were obtained by ModelTest 3.7 (Posada & Crandall 1998). For retinoic acid receptors the Tamura-Nei model TrN+I+G (alpha = 0.7916, p_{inv} = 0.4111; (Tamura & Nei 1993)), for aldh1a enzymes the general time reversible model GTR+I+G (alpha = 1.1961, p_{inv} = 0.2136; (Rodriguez *et al.* 1990)) and for androgen receptors the Hasegawa-Kishino-Yano model HKY+G (alpha = 0.5019, T_{Ratio} = 1.1257; (Hasegawa *et al.* 1985)) was used. ML tree topologies were evaluated by a bootstrap analysis with 500 replicates (Felsenstein 1985). To confirm obtained tree topologies Bayesian analyses were initiated with random seed trees and were run for 1,000,000 generations. The Markov chains were sampled at intervals of 100 generations with a burn in of 1000 generations. Bayesian phylogenetic analyses were conducted with MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001).

Conserved protein domains were identified by searching the Pfam database (http://pfam.sanger.ac.uk/search).

The following sequences were used for the phylogenetic analysis:

aldh1a1: *Homo sapiens* (NM_000689), *Mus musculus* (NM_013467), *Gallus gallus* (NM_204577), *Xenopus laevis* (NM_001087772)

aldh1a2: *Homo sapiens* (NM_003888), *Mus musculus* (NM_009022), *Gallus gallus* (NM_204995), *Xenopus laevis* (NM_001090776), *Danio rerio* (AF315691), *Gasterosteus aculeatus* (ENSGACT00000020927), *Oryzias latipes* (ENSORLT00000010445), *Takifugu rubripes* (NM_001033639), *Tetraodon nigroviridis* (CAAE01013867)

*aldh1a3:*Homo sapiens (NM_000693), *Mus musculus* (NM_053080), *Gallus gallus* (NM_204669), *Xenopus laevis* (NM_001095605), *Danio rerio* (DQ300198), *Gasterosteus aculeatus* (ENSGACT00000018580), *Tetraodon nigroviridis* (GSTENT00012805001), *Takifugu rubripes* (NEWSINFRUT00000155714)

aldh1a1/2/3: *Ciona intestinalis* a (ENSCINT00000016285), *Ciona intestinalis* b (ENSCINT00000016054), *Ciona intestinalis* c (ENSCINT00000016069), *Ciona intestinalis* d (ci0100136702)

rarg: *Homo sapiens* (NM_000964), *Mus musculus* (NM_009024), *Gallus gallus* (X73972), *Notophthalmus viridescens* (X17585)

rarga: *Danio rerio* (NM_131406), *Tetraodon nigroviridis* (GSTENT00024106001), *Takifugu rubripes* (GENSCAN00000028342)

rargb: *Danio rerio* (NM_131399), *Gasterosteus aculeatus* (ENSGACT00000007038), *Takifugu rubripes* (GENSCAN00000013561), *Tetraodon nigroviridis* (GWSHT00007447001)

rarg: *Homo sapiens* (NM_000965), *Mus musculus* (NM_011243), *Gallus gallus* (NM_205326), *Notophthalmus viridescens* (AY847515)
rarg: *Homo sapiens* (NM_000966), *Mus musculus* (NM_011244), *Mesocricetus auratus* (AY046945)
rarga: *Danio rerio* (S74156), *Takifugu rubripes* (GENSCAN00000021740), *Tetraodon nigroviridis* (GSTENT00028047001), *Gasterosteus aculeatus* (ENSGACT00000012380)
rargb: *Danio rerio* (NM_001083310), *Gasterosteus aculeatus* (ENSGACT00000000789), *Takifugu rubripes* (GENSCAN00000014750)
ar: *Homo sapiens* (NM_000044), *Mus musculus* (NM_013476), *Gallus gallus* (NM_001040090), *Xenopus laevis* (NM_001090884)
arb: *Gasterosteus aculeatus* (AY247207),(AY247206),*Oryzias latipes* (NM_001122911), *Tetraodon nigroviridis* (CAAE01014703), *Takifugu rubripes* (GENSCAN00000027349), *Oreochromis niloticus* (AB045212), *Gambusia affinis* (AB182329)
arb: *Gasterosteus aculeatus* (GENSCAN00000022206),*Oryzias latipes* (NM_001104681), *Tetraodon nigroviridis* (CAAE01014998), *Takifugu rubripes* (GENSCAN00000026438), *Oreochromis niloticus* (AB045211), *Gambusia affinis* (AB174849)
pgr: *Homo sapiens* (NM_000926), *Mus musculus* (NM_008829), *Gallus gallus* (NM_205262)

Results

Isolation of RA- and androgen signaling pathway components from *X. hellerii*

To test whether RA is involved in the development of the gonopodium, we screened a *X. hellerii* cDNA library that we had constructed from developing swords and gonopodial tissue, for orthologs of RA synthesizing enzymes (*aldh1as*) and *retinoic acid receptors* (*rars*). We amplified a 721 bp fragment of a putative *aldh1a2* ortholog (FJ372848) that codes for a 240 aa sequence of the protein. Phylogenetic reconstruction of *aldh1a* enzymes, using coding sequences, confirmed that the fragment is a partial sequence of the *X. hellerii aldh1a2* ortholog (Figure 2.2A). In addition, four cDNA fragments were cloned, that encompassed parts of the open reading frames and the complete 3'-UTR sequences of two *rar-g* orthologs. Phylogenetic reconstruction of *retinoic acid receptors*, using coding sequence, confirmed that we cloned partial sequences of *X. hellerii rar-ga* (FJ372849) and *rar-gb* (FJ372850) (Figure 2.2B). The partial *rar-ga* and *rar-gb* sequences code for parts of the protein including most of the zinc finger DNA binding domain and the complete nuclear hormone receptor ligand-binding domain.

To examine the expression of the androgen receptors involved in metamorphosis of the gonopodium, we screened the cDNA library by filter screening and isolated two androgen receptor cDNAs. A 2418 bp cDNA clone codes for 596 aa of *androgen receptor a*, including

the zinc finger DNA binding domain and the complete nuclear hormone receptor ligand-binding domain and the 3'UTR. A second 3867 bp cDNA clone was identified as *androgen receptor b* and covers the complete coding region and 3'-UTR sequence as well as parts of the 5'-UTR sequence. We detected the same two conserved domains in the 756 aa protein sequence as in the first clone. Phylogenetic reconstruction using the amino acid sequence translations from cDNA fragments coding for the zinc finger and the ligand binding domains confirmed the cDNAs as *androgen receptor a* (FJ372851) and *androgen receptor b* (FJ372852) orthologs, respectively, of *X. hellerii* (Figure 2.3).

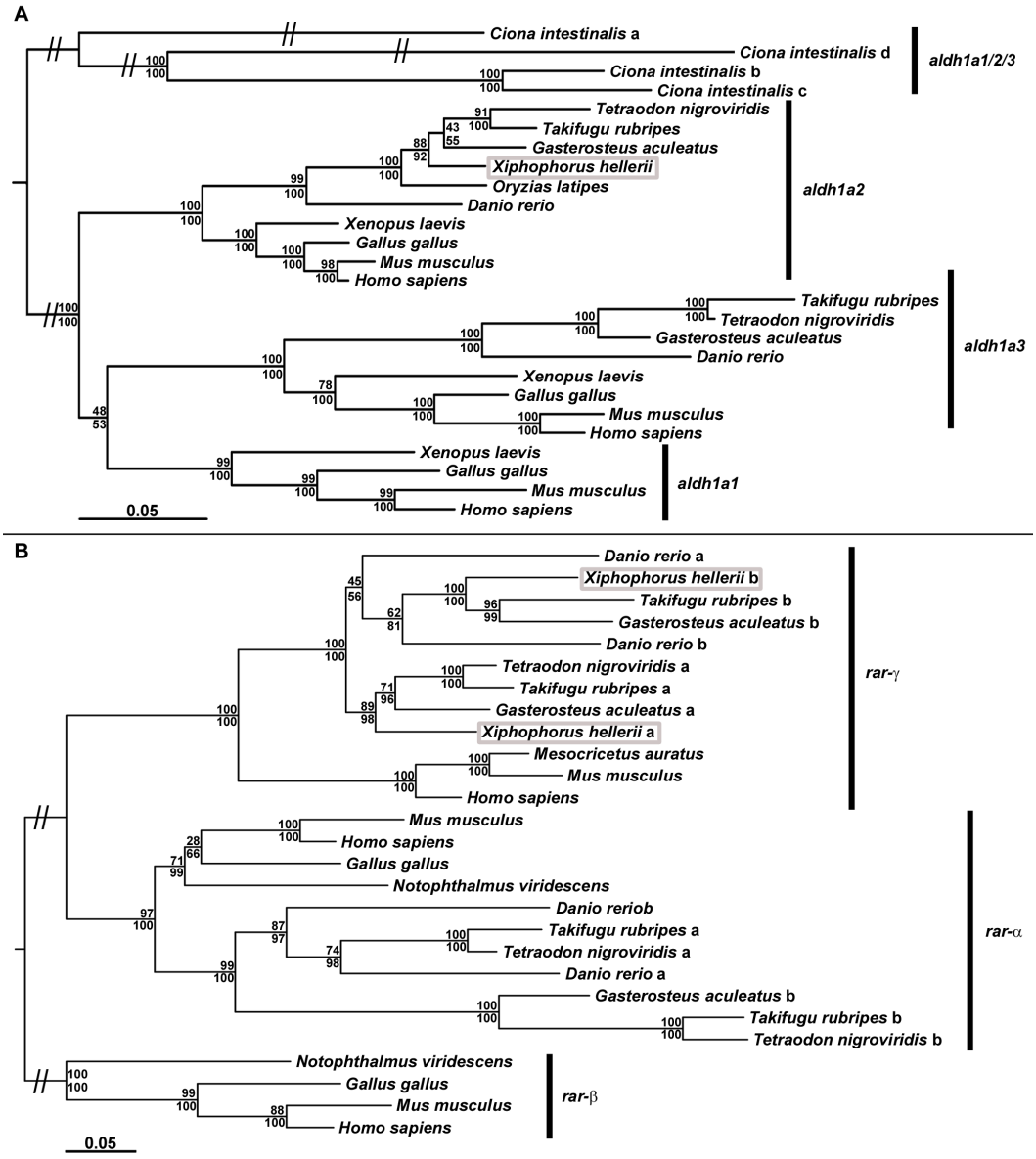

Figure 2.2 Phylogenetic reconstruction of *aldh1a* and *rar* sequences.

Phylogenetic analysis of chordate *aldh1a* enzymes (A) and *retinoic acid receptors* (B) using PhyML (upper values) and MrBayes (lower values). For the analysis the coding regions of *aldh1a* and *rar* cDNAs were used. The position of the *X. hellerii* orthologs of *aldh1a2*, *rar-ga* and *rar-gb* within the two phylogenies is highlighted (grey box).

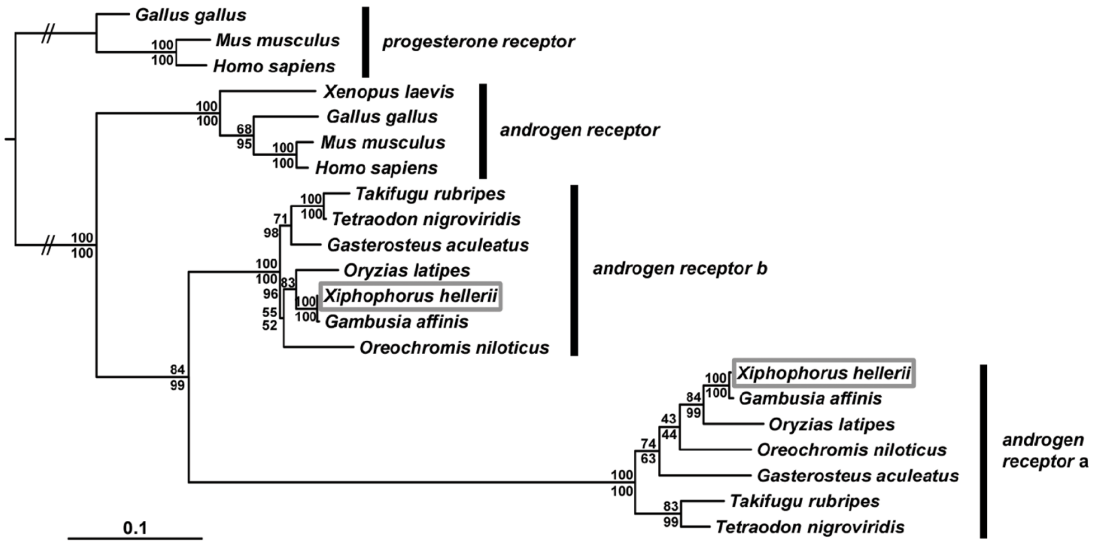

Figure 2.3 Phylogenetic reconstruction of *androgen receptor* sequences.

Phylogenetic analysis of vertebrate *androgen receptors* using PhyML (upper values) and MrBayes (lower values). For analysis the coding regions of *androgen receptor* (*ar*) cDNAs were used. The position of the *X. hellerii ara* and *arb* orthologs within the phylogeny is highlighted (grey box).

RA signaling pathway components are expressed in developing gonopodia

As a first step towards exploring whether RA signaling plays a role in gonopodium development, we analysed the expression of *aldh1a2* and the two *rar*-paralogs. To induce metamorphosis of the pre-adult anal fin, we treated juvenile individuals of *X. hellerii* with 17-α-methyltestosterone and analysed gene expression by *in situ* hybridisation at different stages of gonopodium development. After 5 days of testosterone treatment (dt), *aldh1a2* (Figure 2.4A), *rar-ga* (Figure 2.4B) and *rar-gb* (Figure 2.4C) were up-regulated in the 3-4-5 complex that gives rise to most of the gonopodial structures. In addition, the two *rars* (Figures 2.4B, C) and *aldh1a2* were also expressed in fin rays 6 and 7 that flank the 3-4-5 complex (compare Figures 2.4A, B, C). At 7 dt, when the elongation of the 3-4-5 complex was clearly visible, *aldh1a2* was expressed in a continuous distal stripe that encloses the distal tip of the 3-4-5 complex and several other gonopodial rays (Figure 2.4D). The expression patterns of the *rarg* transcripts at 7 dt appeared to be unchanged compared to 5 dt (Figures 2.4E, F). *In situ* hybridisation on fin sections revealed that *aldh1a2* was expressed in the distal-most mesenchyme of the gonopodial rays (Figure 2.4G). The two *rarg* transcripts showed

overlapping expression with *aldh1a2* in the distal mesenchyme; however, we detected additional expression domains in the lateral mesenchyme (Figures 2.4H, I). Both receptors did not seem to differ in their temporal-spatial expression. At later stages of gonopodium development (18dt), when the first terminal structures, the spines, had started to form, *aldh1a2* remained up-regulated (Figures 2.4J, C). *rar-ga* and *rar-gb* were slightly down-regulated compared to 7 dt (Figures 2.4K, L). In untreated control fins no up-regulation of *aldh1a2* and *rar-g*s was detected (Figure 2.4M-O). The two *rar-gs* were expressed at a basal level (Figures 2.4N, O), whereas *aldh1a2* expression was not detected by *in situ* hybridization (Figure 2.4M). The observed changes in gene expression profiles during gonopodial development suggest that transcriptional activation of *aldh1a2* and the two *rar-g* paralogs correlates with the transformation of the male anal fin into an intromittent organ.

Androgen receptors are expressed in developing gonopodia

Androgen receptor a showed a diffuse pattern of low-level expression in all fin rays of the developing gonopodium (data not shown). In contrast, after 5 days of testosterone treatment (dt) *androgen receptor b* (*arb*) was strongly up-regulated in the distal tip of the 3-4-5 complex and in the inter-ray tissue (Figure 2.5A). After 7 dt, when the 3-4-5 complex was clearly elongated, a similar pattern was detected that differed from the low-level expression in the fin rays that do not participate in gonopodium formation (Figure 2.5B). *In situ* hybridisation on sections of 7 dt gonopodia revealed that *arb* was expressed in a layer of mesenchymal cells underlying the epidermis (Figure 2.5C). The expression domain covers the distal-most mesenchyme, where also *aldh1a2* is expressed, and expands proximally, overlapping with the expression of the two *rar-g* paralogs (compare Figures 2.4G-I and 2.5C). Up-regulation of *arb* in the 3-4-5 complex persisted in later stages of gonopodium development (18 dt) when the first distal structures were present (Figure 2.5D). The expression of *arb* could also be detected in the 3-4-5 complex of untreated control fins, but at lower levels than in treated fin (compare Figure 2.5A to 5E). In addition, expression could be detected in the segment borders when samples were stained for a longer time period (Figure 2.5E).

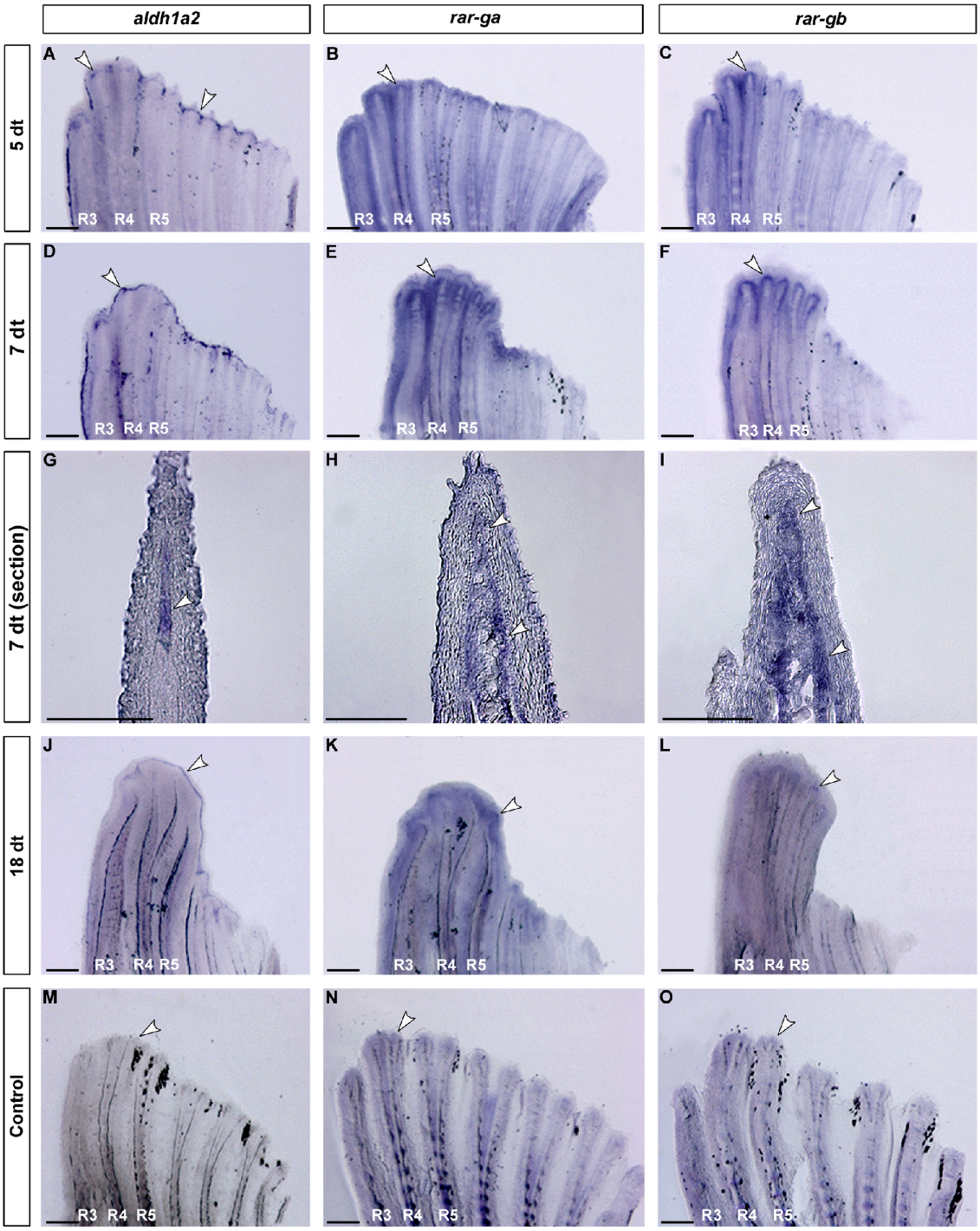

Figure 2.4 Expression of *aldh1a2*, *rar-ga* and *rar-gb* during gonopodium development. *aldh1a2* and both *rarg* paralogs are expressed in developing gonopodia of *X. hellerii*. At 5 (A), 7 (D) and 18 (J) days of testosterone treatment (dt) *aldh1a2* is expressed the distal-most mesenchyme of the main gonopodial rays and extends into rays 6 and 7 (G). *aldh1a2*

expression was not detected under identical conditions in control fins (M). *rar-ga* and *rar-gb* are expressed in an overlapping pattern. At 5 (B, C), 7 (E, F), and 18 dt (K, L) strong expression of both genes could be detected in the gonopodial rays 3-5 and also partly in ray 6 and 7. At 18 dt both genes appeared slightly down-regulated. The expression domains of both genes include the distal-most and more proximo-lateral mesenchymal cells (H, I). No up-regulation was detected in the control fins (N, O). White arrowheads highlight gene expression. (n = 6 for 5 and 7dt, n = 4 for 18 dt, and n = 5 for controls for every probe); scale bars: A-F and J-O: 200 μm; G-I: 100 μm.

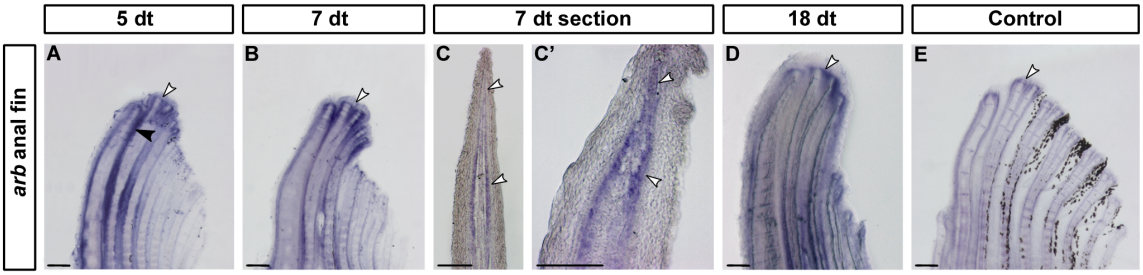

Figure 2.5 Expression of *androgen receptor b* in the developing gonopodium.

Androgen receptor b (*arb*) is expressed in developing gonopodia of *X. hellerii*. At 5 days of testosterone treatment (dt) *arb* is strongly up-regulated in distal tip of the gonopodial rays 3, 4 and 5 and clearly weaker in the rays 6-7 (A). Expression could also be detected in the inter-ray tissue (black arrowhead). This expression pattern persists at 7 (B) and 18 dt (D). Longitudinal sections of anal fins after 7 dt revealed *arb* to be expressed both in the distal and proximal mesenchyme (C, C'). In control fins *arb* is expressed at basal levels (E). White arrowheads indicate gene expression. (n > 10 for every stage and probe; scale bars: A, B, D-F and H, I: 200 µm; C, C', G: 100 µm)

Induction of *aldh1a2* expression is proportional to the rate of metamorphosis

To better understand the dynamics of RA signaling during the metamorphosis of the anal fin towards a gonopodium we examined the regulation of *aldh1a2* expression by real-time quantitative PCR (qPCR) (Figure 2.6). To test whether *aldh1a2* is induced by testosterone in a concentration-dependent manner, we investigated the response to increasing concentrations (2, 5 and 10 µg/l) of 17-α-methyltestosterone. The amount of transcript of *aldh1a2* after 7 days of treatment was increased at all three concentrations of testosterone-treated fish compared to mock-treated controls (Figure 2.6A). The concentrations of 5 and 10 µg/l displayed 2.8- to 3.9-fold induction in *aldh1a2* expression ($p < 0.05$, ANOVA), whereas the concentration of 2 µg/l showed 2.5-fold induction with moderate p-value ($p = 0.052$, ANOVA). Linear regression analysis based on the qPCR analysis confirmed the association of the concentration of 17-α-methyltestosterone with the level of *aldh1a2* gene expression ($r^2 = 0.461$, $p = 0.015$) and supports the notion that *aldh1a2* expression levels correlate with an increase of androgen signaling. In addition, we observed that the higher testosterone concentrations (5 and 10 µg/l) induced more developed gonopodia in terms of the average number of segments that have developed in rays of the 3-4-5 complex when compared to the low concentration (Figure 2.7). Thus under our experimental regime the maximum number of

segments is already induced at a concentration of 5 µg/l, whereas a further increase in concentration has no additional effects on segment number.

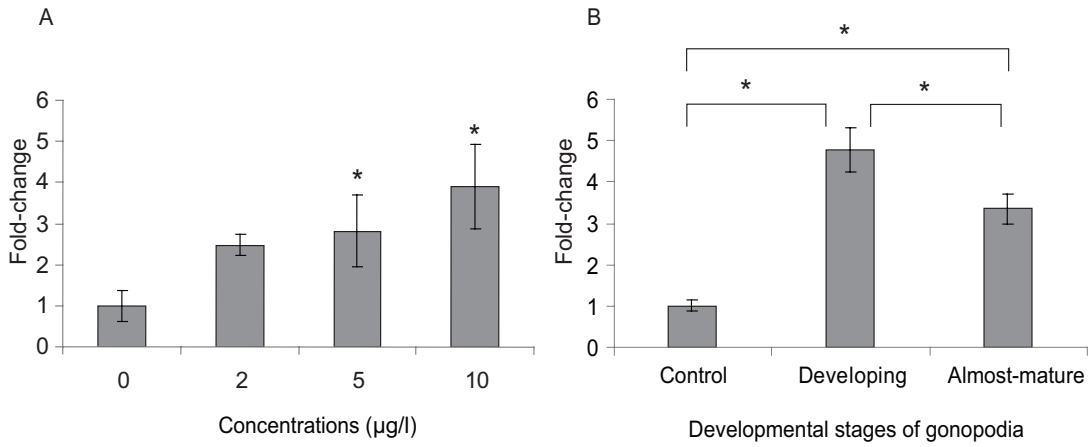

Figure 2.6 Increase in *aldh1a2* expression in testosterone-induced gonopodia.

Fold-changes of *aldh1a2* expression in testosterone-induced gonopodia after 7 days of treatment (A) and in naturally developing gonopodia (B). Anal fins of females (n = 5) were used as control group and developing (n = 4) and complete (n = 8) gonopodia from males were used (B). Asterisks indicate statistically significant differences compared to the control ($p < 0.05$, ANOVA). Figure is plotted as means ± SE.

Differentiation of terminal gonopodial ray structures correlates with decreased RA signaling

The development of an artificially induced gonopodium differs from that of a naturally developing gonopodium in that exogenous addition of testosterone leads to a shorter overall length of the gonopodium (Figure 2.1) (Sangster 1948). Therefore, *aldh1a2* gene expression was also investigated in naturally developing gonopodia (Figure 2.6B). Because of large variation and unpredictability in the maturation times in different individuals it was technically impossible to obtain large numbers of males at the exact same stages of natural gonopodium development. Hence, we introduced two categories for the stage of gonopodium development, which we classified as "developing" gonopodia (apparent increase in the length of the 3-4-5 complex) and "almost-mature" (formation of distal structures) gonopodia, as compared to the anal fins of mature females as a control (see details under Experimental Procedures). *aldh1a2* transcripts were considerably increased both in "developing" gonopodia (by 4.8 times) and in "almost-mature" gonopodia (by 3.7 times) (Figure 2.6B).

However, *aldh1a2* expression levels are significantly higher in "developing" compared to "almost mature" gonopodia ($p < 0.05$, ANOVA). These results suggest that *aldh1a2* gene expression is up-regulated in naturally developing gonopodia and is then down-regulated towards completion of the final gonopodial structure.

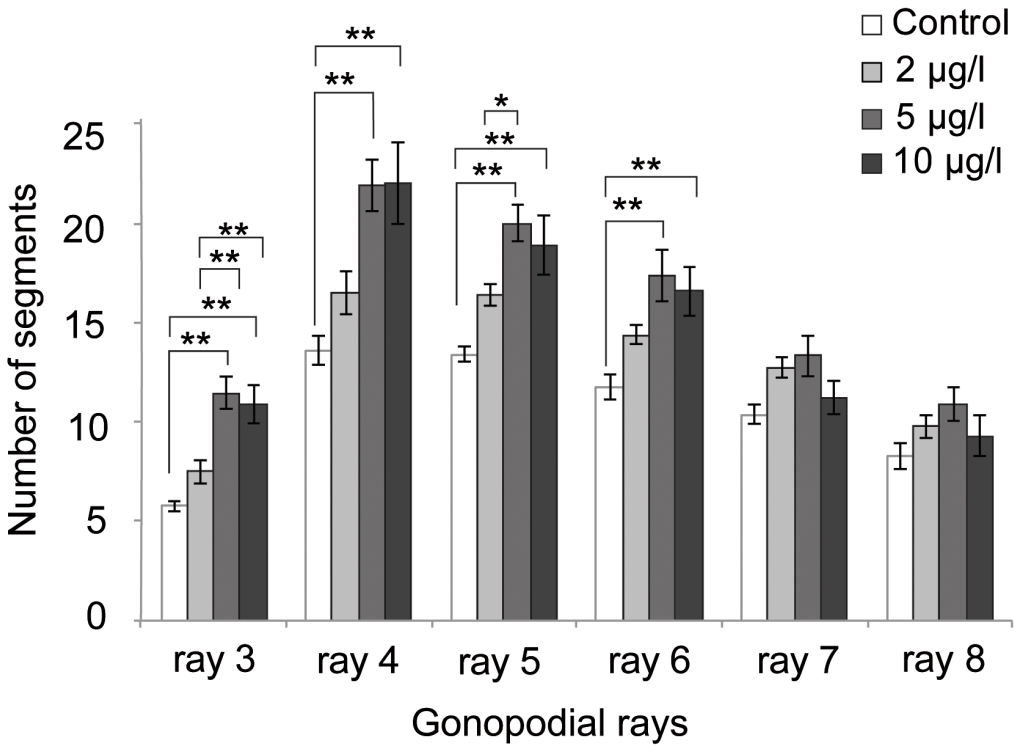

Figure 2.7 Segment numbers of rays 3, 4, 5, 6, 7 and 8 in testosterone-induced gonopodia at day 7 during treatment.

The number of segments of each ray in different concentrations of 17-α-methyltestosterone was measured (n = 7 to 8). Asterisks indicate statistically significant differences (*$p < 0.05$, **$p < 0.01$, rank by Tukey). Figure is plotted as means ± SE.

RA signaling is involved in patterning of the gonopodium

The positive correlation between increases in testosterone levels and *aldh1a2* expression suggested that RA signaling might be a major determinant of gonopodial development. To test this idea more directly, we enhanced RA signaling during testosterone-induced

metamorphosis and examined its effects on gonopodium development. *Xiphophorus hellerii* juveniles were treated twice with 17-α-methyltestosterone to induce gonopodium development, followed by intraperitoneal (IP) injection of *all-trans* RA on days 4 and 6 (see Materials and Methods). For the experiment we chose a 17-α-methyltestosterone concentration of 5µg/l, which we found to be the lowest concentration that led to the maximum increase in segment number. The growth of gonopodial rays was compared between controls (PBS–injected) and RA-injected fish at day 7.

First, we found that the total number of new segments was significantly higher in RA-injected fish relative to control fish in gonopodial rays of the 3-4-5 complex (2.49 segments in RA-injected vs. 1.35 in control, Mann-Whitney, $p < 0.05$) (Figure 2.8A). This increase in segment number is specific for the 3-4-5 complex and was not observed for non-gonopodial rays (rays 6, 7 and 8). Second, the amount of tissue added to each ray as a result of proliferation was increased in gonopodial rays in RA-injected fish. While the lengths of rays 4 and 5 were significantly increased in RA-treated fish compared to control animals (t-test, Mann-Whitney respectively, $p < 0.05$), we found only marginally significant differences in the length of ray 3 (t-test, $p = 0.073$). The length of the non-gonopodial rays 6 and 7 was not significantly increased and that of ray 8 indeed was decreased in RA-injected fish (t-test, $p < 0.05$) (Figure 2.8B). To examine the relative contributions of segmented rays and unsegmented distal tissue to the new tissue added by growth, we measured the former as distance between a reference point and the distal-most segmental border (Figure 2.8C) and the latter as distance between distal-most segmental border and the tip of the ray (Figure 2.8D; see diagram in Figure 2.8E). The total length of newly formed segments (Figure 2.8C) was significantly increased for rays 3, 4, 7 and 8 in RA-injected fish compared to control fish (t-test, $p < 0.05$, $p < 0.01$ respectively). It was increased significantly more in gonopodial rays (rays 3 and 4) than non-gonopodial rays (rays 7 and 8) (Figure 2.8C). No significant differences, however, were found in rays 5 and 6 between the groups (Figure 2.8C). The length of the distal region was also increased for rays 4 and 5 (t-test, $p < 0.05$ and $p = 0.059$ respectively) (Figure 2.8D). This shows that both areas contributed to the increased total length of outgrowth (proliferation) of gonopodia in the RA-injected fish. Overall, an increase in RA signaling during anal fin metamorphosis increases the rates of distal tip proliferation and proximal ray segmentation and suggests that gonopodial development is driven by *aldh1a2*-dependent RA signaling.

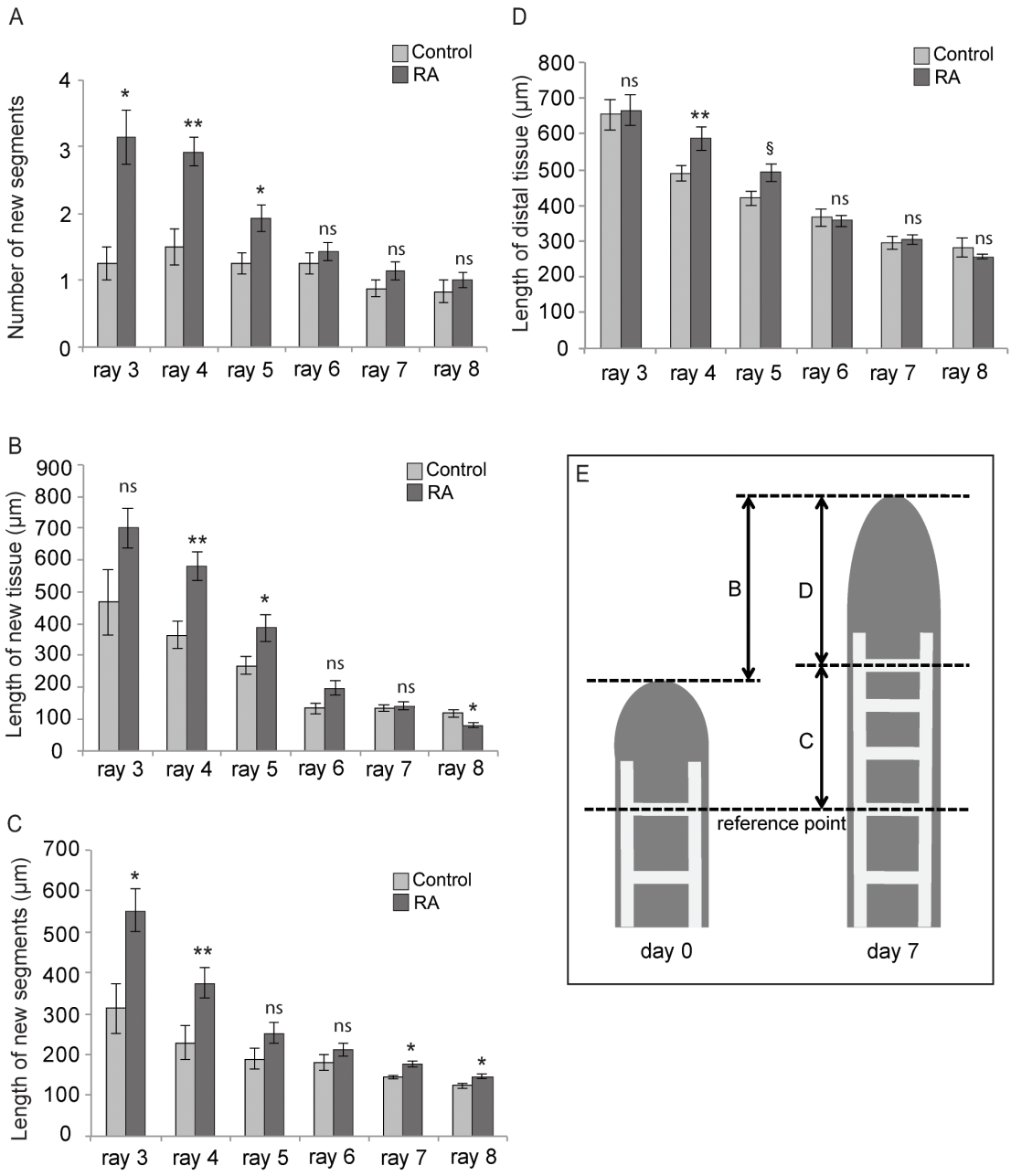

Figure 2.8 Increased activation of RA signaling in testosterone-induced gonopodia affects growth and development.

The number of new segments (A) and the length (in µm) of the tissue added by growth (B) in artificially induced gonopodia were compared in control and RA-injected fish after 7 days of treatment (n = 4 for control and n = 7 for RA). For these measurements, we initially set a reference point, which is the distal most segmental border of the last formed segment at day 0 (before treatment) (E). The distance from the reference point to the tip of developing

gonopodia at day 0 was excluded from the same distance at day 7 (B). The total distance at day 7 in each ray was significantly increased in RA-injected fish (B). The length of new segments (from the reference point to the distal boundary of the last segment at day 7) (C) and the length of tissue of tip of gonopodia (from the distal border of the last segment to the tip of gonopodia at day 7) (D) were measured separately. Both lengths (C, D) were contributed to the increased total length of outgrowth of gonopodia (B). *indicates statistically significant differences compared to the control and § indicates marginal significance (*$p < 0.05$, **$p < 0.01$, $0.05 < {}^{\S}p < 0.08$). Figure is plotted as means ± SE.

Discussion

Although the role of RA signaling in embryonic development and fin regeneration has been studied in several groups of organisms (Maden 2000; Campo-Paysaa *et al.* 2008; Rhinn & Dolle 2012), its role in fin metamorphosis of teleost fish had remained largely unexplored. Gonopodium development differs from embryonic fin development and regeneration in several aspects. First, the gonopodium forms during sexual maturation when its development is triggered by increased synthesis of sex steroids. Second, the gonopodium is a male specific structure that does not develop in females under natural conditions. Third, a specific subset of gonopodial rays develops into a morphologically highly specialized structure with a prominent proximal-distal polarity. Therefore, an investigation of the role of RA signaling pathway in developing gonopodia should provide valuable information for the understanding of its role in postembryonic metamorphosis of fins and the evolution of the gonopodium in poeciliid fish. The current study provides functional evidence that RA signaling is involved in anal fin metamorphosis in the green swordtail, *X. hellerii*.

Up-regulation of *aldh1a2* in hormone-induced gonopodia suggests that RA signaling is involved in gonopodium development

Our expression analyses showed that RA signaling pathway components (*aldh1a2* and the two *rar-g* paralogs) are continuously expressed in testosterone-induced gonopodia at different time points (5, 7 and 18 dt) (Figure 2.4). Expression of *aldh1a2* was up-regulated by testosterone in a concentration-dependent manner (Figure 2.6A). These results suggest that RA signaling plays a role in gonopodium development. It is worth noting that, due to high

levels of exogenous testosterone, hormone-induced gonopodia show alterations in final morphology and developing timing compared to naturally developing gonopodia. Hormone induced gonopodia are shorter (compare Figure 2.1B and 1C) and also possess a reduced number of segments (e.g., 20 for hormone induced ray 3 vs. 38 normal ray 3 adult male, Figure 2.7) compared to naturally developing ones (Grobstein 1942; Sangster 1948). In addition, the developmental process of hormone-induced gonopodia was completed in a much shorter time (18 days) than what is observed for naturally developing gonopodia (up to 6 months). We propose that these differences can be explained by Turner's two-phase model, in which early and late phases of gonopodium development depend on changes in internal testosterone levels. Turner predicted that low concentrations of testosterone at the beginning of maturation would induce outgrowth of the 3-4-5 complexes, whereas high concentrations induce the formation of terminal structures (Turner 1947b). Under this model, terminal segments that contribute to the distal structures could develop immediately in experimentally induced gonopodia due to the premature induction of the second phase by high levels of exogenous testosterone. This view is consistent with earlier observation (Turner 1941a; Grobstein 1942) as well as our own findings (Figure 2.1).

The expression of *aldh1a2* showed temporally and spatially distinct patterns: *aldh1a2* expression was stronger in rays 6 and 7 than in the 3-4-5 complex at day 5 when the gonopodium initiates outgrowth, whereas when the gonopodium is further developed, at days 7 and 18, *aldh1a2* expression is considerably stronger in the 3-4-5 complex region (Figure 2.4). The rise in *aldh1a2* expression in the 3-4-5 complex therefore correlates with the accumulation of sex steroids throughout gonopodium development. In addition, a rise in testosterone causes a concomitant rise in *aldh1a2* expression. These observations suggest that the levels of RA signaling, through the regulation of RA synthesis, are controlled by rising concentrations of sex steroids during gonopodial development.

Naturally developing gonopodia show a dynamic up-regulation of *aldh1a2* expression

Naturally developing gonopodia, in which the different phases of gonopodium development are more distinct, could help to determine the precise roles of RA signaling. The metamorphosis of the anal fin into an intromittent organ involves several developmental events such as fin ray outgrowth, segmentation of new lepidotrichs and development of specialized distal structures like serrae, spines, hooks and claws (Gordon & Rosen 1951). We defined two categories of naturally developing gonopodia, "developing" and "almost-mature",

that may be considered as the first (outgrowth, including addition of new segments) and second phase (formation of distal structures) in Turner's model of gonopodium development, respectively. Up-regulation of *aldh1a2* expression in both phases of naturally developing gonopodia suggests that RA signaling is involved in the outgrowth of the gonopodium in the early phase, as well as the terminalisation in the later phase (Figure 2.6B). Gene expression of *aldh1a2* was significantly increased when gonopodia experienced fin ray outgrowth (Figure 2.6B). Elevated levels of gene expression, albeit reduced, still persisted during terminal development (Figure 2.6B). Thus, dynamic expression of *aldh1a2* is associated both with fin ray outgrowth and formation of terminal structures.

Increased RA signaling promotes gonopodium proliferation and segmental differentiation

To substantiate the idea that the correlation between the dynamics of *aldh1a2* expression and sex steroid signaling is relevant for the regulation of gonopodium formation or growth, we over-activated the RA signaling pathway. In gonopodial rays (3-4-5 complex), intraperitoneal (IP) injection of RA resulted in a significant increase in the gonopodial growth rate (Figure 2.8B) with a strong effect on the segmented rays that are laid down proximally (Figure 2.8C) and milder effects on the size of the proliferating region in the distal tips of fin rays (Figure 2.8D).

The effects of RA on the increase of the growth rate (Figure 2.8B) and on the size of the proliferating distal tip (Figure 2.8D) are specific to gonopodial rays since no significant changes were found in non-gonopodial rays (ray 6, 7 and 8) of RA-injected fish compared to control fish (Figure 2.8). Although significant effects on segment length (Figure 2.8C) were found in both gonopodial and non-gonopodial rays, the amount of increase in gonopodial rays (rays 3 and 4) was much greater than in non-gonopodial rays (rays 7 and 8). Ray elongation in RA-injected fish compared to control fish was 239 μm (ray 3), 147 μm (ray 4), 33 μm (ray 7) and 23 μm (ray 8), respectively (Figure 2.8C). It is interesting to note that the non-gonopodial ray 6 retains high expression of *rar-ga* that is comparable to r3-5 (Figure 2.4E) and can be "forced" to at least form a few additional segments in a situation of artificial episodic testosterone exposure (Figure 2.7). It thus appears as if r6 lacks so far unknown signals that would be required for sustained growth and inclusion in the gonopodium. In summary, RA treatment significantly enhances growth of the 3-4-5 complex, whereas growth in non-gonopodial rays is comparatively moderate. However, we could not completely rule

out the possibility that RA might have effects on outgrowth of non-gonopodial rays that we did not detect during the experiment.

It has recently been shown that enhanced RA signaling promotes mesenchymal proliferation during regenerative growth of the zebrafish caudal fin (Blum & Begemann 2012). Specifically, RA signaling is essential for the proliferation of cells in the blastema, which is located in the distal tips of regenerating rays, by cooperating with Fgf, Wnt/β-catenin and non-canonical Wnt signaling pathways. Our findings suggest that RA signaling fulfils a role in promoting the proliferation of cells in the distal fin ray mesenchyme both in development and regeneration.

The increase in the number of new segments upon RA injection was strong and significant only in the gonopodial rays (Figure 2.8A). This might suggest that RA plays a role in fin segmentation and acts more specifically in gonopodial rays (Figure 2.8A). Together with the increase in segment numbers by increased RA signaling, this treatment also produces slightly smaller segments (reduced by 18 % in RA-injected fish compared to controls; data not shown). Although a direct comparison of average segment sizes requires caution, because control fish produced fewer segments than RA-injected fish, these results imply a potential role for RA signaling in the segmentation process. This could be important for the formation of the small segments that characterize the distal region of naturally developing gonopodia (Figure 2.1B). Interestingly, the mechanism by which RA determines the proximo-distal axis of the developing limb is reactivated during limb regeneration (Mercader *et al.* 2005). Altogether, RA signaling influences the proximodistal patterning not only in embryonic development but also in post-embryonic metamorphosis like developing gonopodia or fin regeneration.

Interaction between androgen and RA signaling during gonopodia development

Our study shows a link between androgen and RA signaling during gonopodium development and suggests that some of the effects of testosterone are mediated by an increase in *aldh1a2* expression and concomitant RA signaling. Gonopodium development is activated by elevated levels of testosterone in poeciliid fish (Turner 1941a; Grobstein 1942; Dzwillo 1962, 1964) and we show that elevated levels of testosterone induce further developed gonopodia. Several molecular studies support this view: (1) expression of genes involved in fin ray growth, such as *msxC*, *fgfr1* and *shh*, is induced by testosterone (Zauner *et al.* 2003; Ogino *et al.* 2004; Offen *et al.* 2008). It has been suggested that androgen signaling acts

upstream of a signaling cascade that results in the activation of downstream effectors, such as Fgf-signaling and *msxC*, to shape the gonopodium (Zauner *et al.* 2003; Offen *et al.* 2008), (2) *androgen receptors* (*ars*) are expressed in the developing gonopodium (this study and Ogino *et al.* 2004) and (3) inhibition of androgen signaling down-regulates gene expression and perturbs gonopodium growth (Ogino *et al.* 2004). We show that *aldh1a2* expression is induced by testosterone in the anal fin (Figure 2.4) and its levels are correlated with the concentration of testosterone (Figure 2.6A), suggesting that androgen signaling regulates *aldh1a2* expression. We show that expression of *arb* overlaps with that of *aldh1a2* in the distal-most mesenchyme of the 3-4-5 complex and also in the anal fin rays 6 and 7. The *arb* gene is expressed in the segment boundaries of two adjoining segments in the gonopodium, which only becomes visible after prolonged staining (data not shown). The function of *arb* in the segment boundaries is unknown, but it is possible that *arb* is directly involved in the establishment or maintenance of segment boundaries. Under this scenario increased levels of testosterone and RA would promote fin ray segmentation, which is supported by the outcomes of RA injection experiments. In addition, a role of *ara* in *aldh1a2* regulation cannot be ruled out, since *ara* is also expressed in the developing gonopodium, though in a diffuse pattern. *arb* expression also overlaps with that of the two *rar-g* paralogs. Experiments in rats confirmed a regulation of *rar-g* by androgen signaling in several tissues (Huang *et al.* 1997). Several studies suggest that there may be crosstalk between RA- and sex steroid signaling pathways during embryonic development of sexual organs or reproductive systems, e.g., in the development of imposex in gastropod females (Stange *et al.* 2012), the development of mammalian external genitalia (Cohn 2011) and sexually dimorphic anal fin, which is an electric discharge organ in electric fish (Herfeld & Moller 1998).

We propose that the sex steroid-controlled metamorphosis of the anal fin towards a gonopodium is mediated by an increase in RA signaling. In support of this idea, we show that *arb* (and *ara*) expression overlaps with *aldh1a2* and *rarg* expression in the growing tips of anal rays that are eventually incorporated into the mature gonopodium. Importantly, increased androgen signaling in artificially and naturally induced gonopodia significantly up-regulates *aldh1a2* expression (RA synthesis) and increased RA signaling in artificially induced gonopodia dramatically improves proliferation and fin ray segmentation in the gonopodium. The molecular mechanisms of gonopodium development are thought to be evolutionarily conserved, despite some variation in its morphology (e.g. terminal structures) between species (Turner 1941a; Gordon & Rosen 1951). RA is a well-known key morphogen

during embryonic development and our work highlights a role for RA signaling in post-embryonic metamorphosis.

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Chapter 3. Transcriptomics of two evolutionary novelties: how to make a sperm-transfer organ out of an anal fin and a sexually selected "sword" out of caudal fin

Ecology and Evolution 5:4

Transcriptomics of two evolutionary novelties: how to make a sperm-transfer organ out of an anal fin and a sexually selected "sword" out of a caudal fin

JI HYOUN KANG[1,2], TEREZA MANOUSAKI[1,3], PAOLO FRANCHINI[1], SUSANNE KNEITZ[4], MANFRED SCHARTL[4,5] AND AXEL MEYER[1,2,*]

[1]*Lehrstuhl für Zoologie und Evolutionsbiologie, Department of Biology, University of Konstanz, Universitätsstraße 10, 78457 Konstanz, Germany*

[2]*Konstanz Research School Chemical Biology, University of Konstanz, Konstanz, Germany*

[3]*Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Heraklion, Greece*

[4]*Physiological Chemistry, Biozentrum, University of Würzburg, Am Hubland, and*[5]*Comprehensive Cancer Center, University Clinic Würzburg, Josef Schneider Straße 6, 97074 Würzburg, Germany*

Email addresses:

JK: jihyoun.kang@uni-konstanz.de

TM: tereza@hcmr.gr

PF: paolo.franchini@uni-konstanz.de

SK: Susanne.kneitz@uni-wuerzburg.de

MS: phch1@biozentrum.uni-wuerzburg.de

AM: axel.meyer@uni-konstanz.de

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Correspondence: Axel Meyer; Lehrstuhl für Zoologie und Evolutionsbiologie, Department of Biology, University of Konstanz, Universitätsstrasse 10, 78457 Konstanz, Germany; +49 (0)7531 88 4163; axel.meyer@uni-konstanz.de

Running tittle: Transcriptomics of Male-specific Traits in Swordtail Fish

Abstract

Swords are exaggerated male ornaments of swordtail fishes that have been of great interest to evolutionary biologists ever since Darwin described them in the Descent of Man (1871). They are a novel sexually selected trait derived from modified ventral caudal fin rays and are only found in the genus *Xiphophorus*. Another phylogenetically more widespread and older male trait is the gonopodium, an intromittent organ found in all poeciliid fishes, that is derived from a modified anal fin. Despite many evolutionary and behavioral studies on both traits, little is known so far about the molecular mechanisms underlying their development. By investigating transcriptomic changes (utilizing a RNA-Seq approach) in response to testosterone treatment in the swordtail fish, *Xiphophorus hellerii*, we aimed to better understand the architecture of the gene regulatory networks underpinning the development of these two evolutionary novelties. Large numbers of genes with tissue-specific expression patterns were identified. Among the "sword genes" those involved in embryonic organ development, sexual character development and coloration were highly expressed, while in the gonopodium rather more morphogenesis-related genes were found. Interestingly, many genes and genetic pathways are shared between both developing novel traits derived from median fins: the sword and the gonopodium. Our analyses show that a larger set of gene networks was co-opted during the development and evolution of the 'older' gonopodium than in the 'younger', and morphologically less complex trait, the sword. We provide a catalogue of candidate genes for future efforts to dissect the development of those sexually-selected exaggerated male traits in swordtails.

Introduction

Exaggerated male-specific traits, or exaggerated secondary sexual characters, are known from many animals and are of great interest to evolutionary biologists. For example, the Peacock's tail, the eye-span of stalk-eyed flies, and the feather ornaments of flycatchers are all believed to be products of inter-sexual selection, as they are attractive to females (reviewed in Andersson 1994). Females are known to base their mate choice on those traits and this will tend to drive their evolution towards becoming more and more exaggerated (reviewed in Andersson 1994).

The "sword" in the genus *Xiphophorus* (Family: Poeciliidae) is a famous example that was already known to Darwin (1871) of a sexually selected male-specific trait. This trait is favored by females although the preference is not fixed in all species (Wong & Rosenthal 2006). The sword is composed of extended colorful ventral caudal fin rays and it characterizes several species of the genus *Xiphophorus*. Species of this genus that carry swords are called swordtails, while non-sworded species are colloquially referred to as platyfish. Within swordtails, the swords vary: some species have very long extended colorful swords that can be longer than the body of the males, whereas others have only short and colorless ventral protrusions in the caudal fin (Rosen 1960; Kallman & Kazianis 2006). Some, swordtails, that also tend to have more slender bodies than platies, e.g., *X. pygmaeus*, and *X. continens*, do not have much of a sword at all (Meyer *et al.* 1994; Meyer 1997). In several *Xiphophorus* species, females prefer males with longer swords and even females in platyfish, whose males do not have swords, are attracted to con-specific males with artificial longer swords and sworded males of other species over their own non-sworded conspecific males (Gordon & Rosen 1951; Basolo 1990b, 1991, 1995a, b). The sword has evoked important questions relating to its origin and subsequent evolution such as the pre-existing female bias hypothesis (Basolo 1990a, 1995b), and the role of this sexually selected trait in hybridization-driven speciation in this fish group (Meyer *et al.* 1994, 2006; Jones *et al*. 2012, 2013; Kang *et al.* 2013). Although a hybrid-origin of species might be expected to be rare, in fish other instances are known, for example also from cichlid fish (Rüber *et al*. 2001). Moreover, one might expect that speciation is accompanied by transcriptomic changes in addition to changes in copy numbers of genes and positive selection acting (Steinke *et al.* 2006; Elmer *et al.* 2010). Recent comprehensive phylogenetic analyses, using different types of molecular markers, have consistently revealed that this evolutionary novelty has arisen once during the diversification of the genus *Xiphophorus* from ancestral poeciliids, but,

interestingly, has been independently lost multiple times (Meyer *et al.* 1994, 2006; Meyer 1997; Kang *et al.* 2013).

Another male specific trait found in all species in the genus *Xiphophorus* is the gonopodium. It is derived from another modified median fin, the anal fin and is made up by its heavily modified rays 3, 4 and 5. They are transformed into this sperm-transferring intromittent organ that is used in copulation in the viviparous poeciliid fish family (Langer 1913; Parenti 1981). While the sword is an evolutionary innovation that is exclusively restricted to the genus *Xiphophorus* (a colorless "sword" is also found in *Poecilia petenensis*), the gonopodium is an evolutionarily older and phylogenetically more widespread trait that is found in all poeciliid fishes. The gonopodium develops species-specific terminal structures such as hooks, spines and a claw, that are believed to serve as a natural mating barrier (in a lock-and-key type) between species, although the proposed prezygotic isolating mechanism has not been shown to act particularly stringently and hybridization is known to occur among some closely related species (Gordon & Rosen 1951; Rosen & Bailey 1963).

Despite numerous phylogenetic and behavioral studies that aimed to study the evolutionary history and behavioral roles in determining mating success of the sword and gonopodium, much less is known about the genetic basis underlying the development of these traits. Interestingly, the induction of the development of the gonopodium by exogenous testosterone has been demonstrated in juveniles, adult females (Grobstein 1947, 1948; Zauner *et al.* 2003; Offen *et al.* 2009, 2013) and even another poeciliid fish, *Gambusia affinis* (Turner 1947; Angus *et al.* 2001; Ogino *et al.* 2004). Testosterone increases outgrowth of the gonopodium in a concentration-dependent manner in *X. hellerii* (Offen *et al.* 2013). Exogenous testosterone can also induce swords or sword-like protrusions from ventral caudal fins in several swordtail and platyfish species (Dzwillo 1964; Zauner *et al.* 2003; Yanong *et al.* 2006). All these pieces of evidence imply that androgen signaling is involved in the hormone induced sword and gonopodial development (Offen *et al.* 2013). However, the signaling pathways have not been investigated at the molecular level and our previous work aimed to find other molecular mechanisms potentially involved in the development of those two male specific traits. Candidate gene approaches revealed that *msxC* and *fgfr1* are up-regulated in developing sword and gonopodium under testosterone treatment (Zauner *et al.* 2003; Offen *et al.* 2008). In a previous study using suppressive subtractive hybridization (SHH), genes or pathways related to sword and gonopodial development were found and we discovered that over 100 genes are involved during the development of both traits in *X.*

hellerii (128 genes were up- and down-regulated in the development of sword and gonopodium) (Offen *et al.* 2009). However, these findings were based on a cDNA library of *X. hellerii,* and differentially expressed genes were detected in the pooled gonopodium and sword tissues. Gonopodium- or sword-specific genes could hence not be discriminated. Exploring gene expression in the development of the gonopodium and the sword separately to determine their specific roles in terms of developmental molecular mechanisms was the aim of this study.

Evolutionary novelties: their evolutionary origins and their developmental bases

Identification of the genes or pathways regulating the transformation of the anal and caudal fin into a gonopodium and the development of a sword during sexual maturation provide a key step to understanding the molecular mechanisms leading to evolutionary innovations such as gains or losses of the sword or intraspecific variation in gonopodium morphology. How evolutionary novel traits arise remains an open question in evolutionary biology. There are several definitions for 'novel traits' or 'evolutionary novelties', yet they are generally seen as structures that are neither homologous to any structure in ancestral species nor homonomous to any other structures of the same organism (Müller & Wagner 1991). Evolutionary novelties can be categorized based on their novel functional capabilities (e.g., flight or vision) or structural elements (e.g., hair and horn in mammals, scales in reptiles) by focusing on the developmental origin of novel body parts (Wagner & Lynch 2010). The investigation of the origin and divergence of the novel gene regulatory networks contributing to morphological innovations would thereby also allow to better understand both their unique developmental and evolutionary identities (Wagner & Lynch 2010).

Here, we performed a transcriptome-wide expression analysis to study the molecular pathways involved in the hormone induced sword and gonopodium development in *Xiphophorus hellerii*. Through RNA-Seq we identified a large number of differentially expressed genes during the development of the sword and the metamorphosis of the anal fin into a gonopodium. These analyses help to increase our understanding of the molecular processes underlying the ontogeny and phylogeny of both of the sword and the gonopodium and provide the foundation for future studies on the molecular mechanisms that led to the evolutionary origin of these evolutionary key innovations.

Materials and Methods

Fish and hormone treatments

To study the molecular pathways involved in the development of sword and gonopodium in *Xiphophorus hellerii,* we testosterone treated immature juveniles to reach the same ontogenetic stages of the development of sword and gonopodium. We chose this experimental design because the timing of sword and gonopodium during sexual maturation and consequent development of secondary sex characters vary among individuals. This approach allowed for better capturing of transcriptional changes during the development of both of those traits by hormonally inducing identical development in all individuals. It is known that no sex-related morphological differences are found in hormonally induced swords in immature juveniles (Dzwillo 1964; Zander & Dzwillo 1969).

Pregnant females of *Xiphophorus hellerii* (Konstanz laboratory strain) were taken from stocks kept at the animal research facility at the University of Konstanz. The fish were maintained on a 12h:12h light:dark cycle at 24°C in 110-liter, densely planted aquaria and were fed with TetraMin flake food and *Artemia* nauplii. A single pregnant female was chosen and kept separately in a 40-liter tank until she gave birth and her juveniles (i.e., one brood) were raised up to three months of age under the same conditions. Juvenile individuals (n = 8; total length: 2.5-2.7 cm) from the same brood were divided into two groups: testosterone-treatment (n = 4) and mock-treatment (n = 4). 17-α-methyltestosterone dissolved in ethanol was added to the water of the treatment group. Either testosterone or mock treatment (ethanol) was repeated at day 4 after the initial treatment to maintain the effect of testosterone. We chose a final concentration of 10 µg /l of testosterone since at this concentration the induction of sword/gonopodium development was successful in our other previous studies (Offen *et al.* 2008, 2009, 2013). A short period of time for treatment (e.g., 1-2 days or even shorter) might be enough for the detection of gene expression differences that are involved in the induction of sword/gonopodium development (more direct target). Although the timing of onset of expression of genes related to sword and gonopodium development after testosterone treatment has not yet been explored in detail. The changes in the expression level of many genes in the fin rays in the hormone treated fish were detected at 5 days of treatment previously (Offen *et al.* 2009). Furthermore, morphological changes (i.e., outgrowth of fin rays) of anal (gonopodium) and ventral caudal (sword) fins in the hormone-treated fish became clearly visible at 5 days.

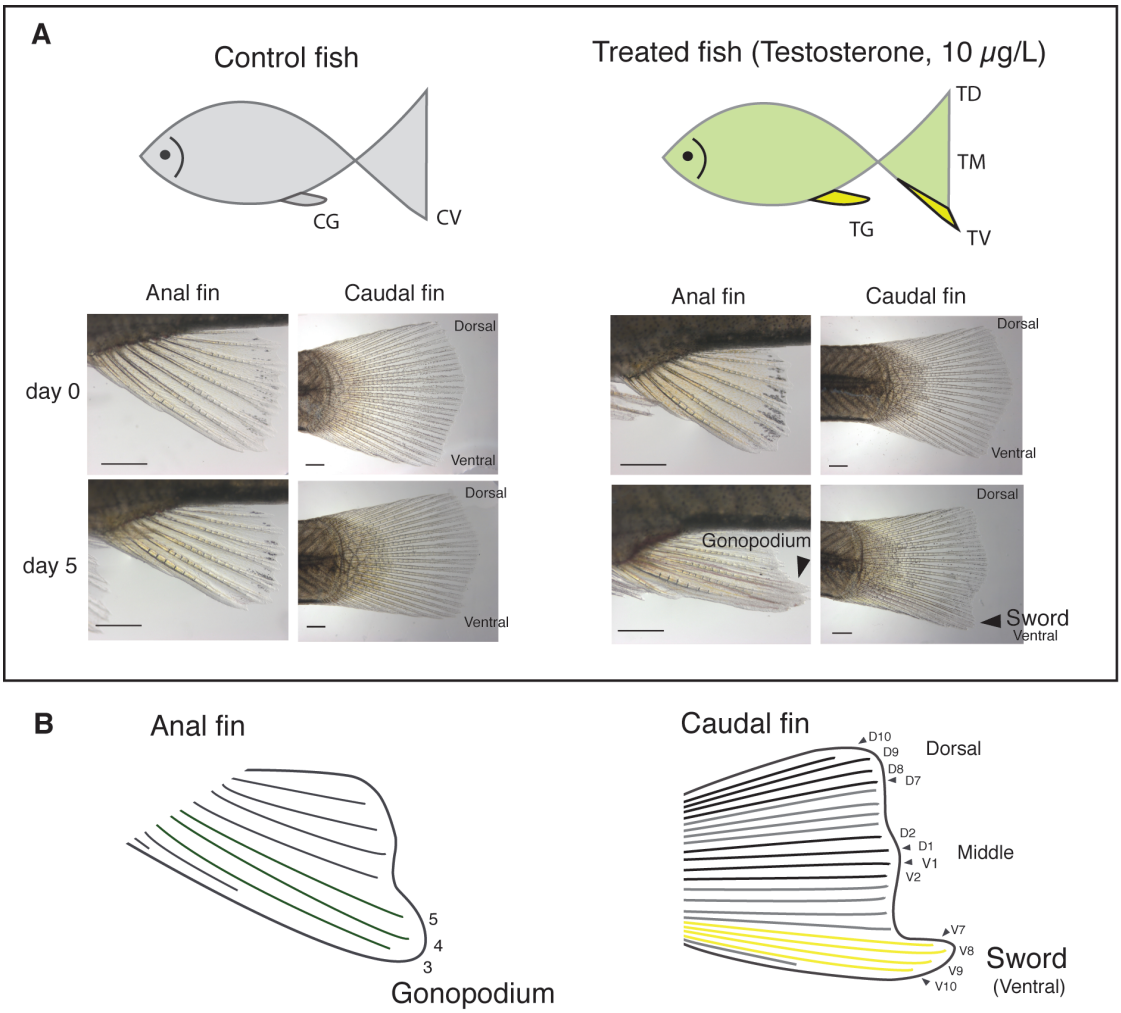

Figure 3.1 Anal and caudal fins in the control and treated fish at day 0 and day 5 (A) and details of fin rays in the developing sword and gonopodium (B).

(A) In the control fish, no difference between anal fin (CG) and ventral caudal fin (CV) was observed at day 0 and day 5 (scale bar = 1mm). In testosterone treated fish, initiation of the transformation into a gonopodium (TG) and sword (TV) was apparent at day 5. (B) The gonopodium developed from rays 3, 4 and 5 in anal fin and the sword developed from V7-V10 (ventral). Tissues from dorsal rays (D7-D10) and middle rays (V1, V2, D1 and D2) were used for RNA sequencing. * T stands for treated, C for non-treated (control), V for ventral caudal rays (sword), M for middle caudal rays, D for dorsal caudal rays.

At day 5 fish were anaesthetized by incubation in a solution of 0.08 mg/ml tricaine (3-aminobenzoicacid-ethylester-methanesulfonate; Sigma, Munich, Germany). Anal (rays 3-5) and dorsal (D7-V10), middle (D1, D2, V1, and V2) and ventral (V7-V10) rays of the caudal fin from testosterone-treated fish were amputated using a sterile razor blade. Only the anal fin (rays 3-5) and ventral (V7-V10) rays of caudal fin were used for mock-treated control fish. The structures of fin rays used in the transcriptomic experiments are shown in Figure 3.1.

RNA extraction

Total RNA of each fin tissue was isolated with Trizol (Invitrogen, Darmstadt, Germany). Tissues were homogenized using pestles and chloroform extraction. RNA was further purified using RNeasy columns (Qiagen, Stockach, Germany). Then on-column DNase treatment was performed according to the manufacturer's protocols (Qiagen, Stockach, Germany). In additional washing and drying steps we washed columns twice with 80% EtOH to remove all traces of salt and ethanol, and spun them dry for 5 min. RNA was eluted in RNase and DNase-free water. RNA purity was assessed by a Nanodrop (Thermo Scientific, Wilmington, Germany) and RNA integrity was assessed using a Bioanalyzer 2100 (Agilent, Waldbronn, Germany).

Library construction and sequencing

Total RNAs recovered from the tissue of dorsal/middle/ventral caudal and anal fin rays in testosterone treated and non-treated control fish were subjected to high-throughput transcriptome sequencing (RNA-Seq). For testosterone-treated fish, 16 cDNA libraries (four tissues x four individuals) were constructed: treated dorsal caudal fin (TD), treated middle caudal fin (TM), treated ventral caudal fin ("developing sword": TV) and treated anal fin ("developing gonopodium": TG) (see Figure 3.1). For control fish, 8 cDNA libraries (two tissues x four individuals) were constructed only from two tissues including untreated-ventral caudal fin (CV) and untreated anal fin (CG) (see Figure 3.1). Sequencing libraries were constructed using the Illumina TruSeq RNA sample preparation kit (Low-Throughput protocol) according to the manufacturer's instructions (Illumina, San Diego, USA). Briefly, 500 ng of RNA was subjected to mRNA selection using poly-T oligo-attached magnetic beads followed by chemical fragmentation (5 min, 94°C). The cleaved RNA fragments were then copied into first strand cDNA using SuperScript II reverse transcriptase (Invitrogen, Darmstadt, Germany) and Illumina proprietary random hexamer primers. After second strand

synthesis using Illumina-supplied consumables, the cDNA was amplified with reagents of the same kit according to the manufacturer's protocols and ligated to barcoded adapters. The final libraries were amplified using 15 PCR cycles. Quality assessment of the libraries was performed on a Bioanalyzer 2100 (Agilent, Waldbronn, Germany) and the quantification was carried out in the Qubit 2.0 fluorometer (Life Technologies, Darmstadt, Germany). The 24 barcoded samples were equimolar-pooled and the same pool was loaded on three different lanes of an Illumina flowcell in order to obtain technical replications as well as considerable sequencing depth. Paired-end sequencing of clustered template DNA was performed in the University of Konstanz Genomics Center (GeCKo) on a Genome Analyzer IIx using four-color DNA sequencing-by-synthesis (SBS) technology with 151 cycles (72 cycles for each paired-read and seven cycles for the barcode sequences).

Raw Reads and quality filtering

After sequencing we obtained 272,288,416 raw reads that were quality-controlled before assembly, read mapping and downstream analyses. First, the remaining adapters were removed with SeqPrep (https://github.com/jstjohn/SeqPrep) and the overlapping paired-reads were merged. Quality of the sequences was assessed with FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) and the reads were trimmed further in CLC Genomics Workbench v4.9 (CLC bio, Aarhus, Denmark). Low-quality reads (CLC parameter 'limit' set to 0.05) and reads shorter than 20 nucleotides were excluded. Finally, we obtained a total of 201,148,252 filtered reads (5,655,070 - 11,861,043 reads per sample) with a mean length of 53 nucleotides.

Transcriptome assembly

Two strategies were employed for producing the assembly: a *de novo* assembly with Velvet 1.2.07 /Oases 0.2.08 (Zerbino & Birney 2008; Schulz *et al.* 2012) and a 'reference-based' assembly using the genome of the closely related species *X. maculatus* as a reference (*Xiphophorus_maculatus*-4.4.2, GenBank Assembly ID: GCA_000241075.1) (Schartl *et al.* 2013). For the *de novo* assembly we used a series of kmer values (21-59 with a step of 2) and merged the produced assemblies as described in the Oases manual. We obtained 23,047 loci (including 247,959 transcripts). The reference-based assembly was produced by aligning the reads to the *X. maculatus* genome using Bowtie2 (Langmead & Salzberg 2012) and the transcripts were produced using Cufflinks 2.0.2 (Trapnell *et al.* 2012). The resulting

reference-based assembly contained 47,812 transcripts assigned to 41,360 different loci. To evaluate the two independent assemblies, a BLASTX (Altschul *et al.* 1997) search was conducted against the stickleback protein dataset of Ensembl v68 (Flicek *et al.* 2012) with e-value cut-off of 10^{-6}. The comparison showed that the reference-based assembly had significant similarities with more stickleback protein-coding genes (15,081) than the *de novo* assembly (13,820). Thus, we chose the reference-based assembly for downstream analyses. To further evaluate the completeness of our transcriptome, we ran CEGMA (Core Eukaryotic Genes Mapping Approach) to search for a set of 248 core proteins that are known to be present in a wide range of species (Parra *et al*. 2007). The results were then compared with those obtained by an independent CEGMA run using as reference the most comprehensive transcriptomic resource publicly available for the genus *Xiphophorus,* the *X. maculatus* cDNA dataset (Ensemble v77) (Cunningham *et al.* 2014).

Differential expression

For obtaining gene-wise mapping results, we kept the longest transcripts per locus against which we mapped the reads again for each sample with Bowtie2. Read counts were obtained through the software SAMtools (Li *et al.* 2009). In total, 188,298,275 reads were uniquely mapped (ranging from 5,069,023 to 10,764,803 per sample). The differential expression analyses were conducted in DESeq 1.10.1 (Anders & Huber 2010). Differential expression was tested among all different tissue types with or without testosterone treatment with FDR adjusted (Benjamini & Hochberg 1995) p-value threshold of 0.05. Blast2GO (Conesa *et al.* 2005) was used to functionally annotate the genes. The differentially expressed (DE) loci were annotated after a BLASTX search against the NCBI *nr* database (e-value < e^{-6}, annotation cut-off > 55, GO weight > 5) and assignment of the corresponding gene ontology (GO) terms. Enrichment analyses, using the Fisher's exact test implemented in Blast2GO, were applied to identify significantly over-represented GO terms comparing DEG sets to the whole assembly as a reference. Pathway analysis was performed using DAVID (Huang da *et al.* 2009).

Results

Exogenous testosterone-driven development of the sword and gonopodium

To explore gene expression profiling of the developing sword and gonopodium at the same ontogenetic stages, we applied a testosterone-treatment on immature juvenile *Xiphophorus*

hellerii of a single brood (see details in Materials and Methods). We observed that all hormone-treated juvenile fish developed both sword-like protrusions from their ventral caudal fins (ray V7-V10) as well as the extension of gonopodial rays from the anal fin (ray V3-V5) after 5 days of treatment. The developing swords and gonopodia were clearly visible after 5 days of treatment (Figure 3.1).

High-throughput transcriptome sequencing of the developing swords and gonopodia

Illumina deep sequencing yielded from 5,655,070 to 11,861,043 reads per sample, with an average of 8,381,177 read per individual (see Table 3.1 for summary). Reference-based assembly suggested the presence of 41,360 different loci (N50: 2,570 bp). Further, 71% of our assembled transcripts had a significant similarity (assessed with BLASTN against *X. maculatus* cDNAs; e-value threshold 10^{-6}) to the previously determined *X. maculatus* transcriptome. Our assembly showed a CEGMA completeness of 93.15% partial and 85.89% complete genes, while the transcriptomic data of *X. maculatus* was composed of 97.98% partial and 88.71% complete genes.

Table 3.1 Summary statistics of Illumina sequencing.

Percentage of the raw reads in each tissue is shown in parentheses.

	Treated				**Non-treated control**	
	Sword (TV)	**Caudal middle (TM)**	**Caudal dorsal (TD)**	**Gonopodium (TG)**	**Sword (CV)**	**Gonopodium (CG)**
Raw reads	47,457,964	46,049,838	45,121,568	42,942,650	41,862,944	48,853,452
Filtered reads	35,055,836 (73.87%)	33,711,045 (73.21%)	33,413,285 (74.05%)	31,841,652 (74.15%)	30,884,763 (73.78%)	36,241,671 (74.18%)
Uniquely Mapped reads	32,917,816 (69.36%)	31,540,697 (68.49%)	31,244,476 (69.25%)	29,898,472 (69.62%)	28,875,936 (68.98%)	33,820,878 (69.23%)
Unmapped reads	2,138,020 (4.51%)	2,170,348 (4.71%)	2,168,809 (4.81%)	1,943,180 (4.53%)	2,008,827 (4.80%)	2,420,793 (4.96%)

Gene expression profiling

Our main goal was to characterize the gene expression profiles of the sword and gonopodial tissues during treatment-induced development. For this, we compared the gene expression patterns between different tissues within the same groups (treated or non-treated) or in the same tissues between the groups in a pairwise manner (Figure 3.2). The total number of differentially expressed genes (DEGs) was determined from all seven comparisons (Table 3.2). The largest number of DEGs was observed in the gonopodium comparison between 'TG' and 'CG' (5,433), while no DEGs were found between 'TM' and 'TD' groups. In the following sections we summarized the analyzed expression patterns of the two developing male-specific organs, the sword and gonopodium separately.

DEGs in the developing sword

To characterize expression profiles of the sword, we compared the gene expression of the ventral ray of the caudal fin (developing sword) with two other tissues of the caudal fin, the middle and the dorsal rays, which retain their typical caudal fin ray morphology during sexual maturation of the fish and its sword development. First, we measured the differences in gene expression patterns for the pairs of ventral rays of the caudal fin between testosterone-treated and untreated fish (TV and CV). A total of 1,784 genes were differentially expressed; 995 genes were up-regulated, while 789 genes were down-regulated in the sword of treated fish (Table S1, Supporting Information). In 1,784 DEGs in the sword (TV and CV), we found that 372 GO terms are over-represented compared to the reference-assembled transcriptome (Table S2, Supporting Information).

To identify which genes were differentially expressed specifically in the sword (TV) we further analyzed the gene expression in the other two tissues of the caudal fin, the middle (TM) and the dorsal rays (TD). The comparison between ventral and middle rays in treated fish (TV and TM) revealed 190 DEGs, whereas that between ventral and dorsal rays (TV and TD) found only 86 DEGs (Figure 3.2). Three independent pair-wise comparisons resulted in 50 common DEGs (48 up-regulated and two down-regulated genes). This limited gene set can be considered as the sword-specific expression profile (Table 3.3; Figure 3.2, Venn diagram; Figure 3.3, Heat map). To investigate functional classifications of sword-specific DEGs we performed a functional enrichment analysis on the limited gene set compared to the assembled transcriptome (Figure 3.3). 15 functional categories were significantly over-

represented (FDR < 0.05) with gonadotropin secretion and follicle-stimulating hormone secretion being the most significantly over-represented (Table S3, Supporting Information).

Pathway analysis and functional annotation results based on the comparison between the sword (TV) and dorsal caudal (TD) fin in the treated fish showed that two of the up-regulated coloration related pathways are over-represented in the developing sword. One is tyrosine metabolism (has00350) and the other is melanogenesis (has04916) (See Figure S1, Supporting Information). Moreover, the functional annotation results revealed that many of the coloration-related GO terms such as melanosome, melanogenesis, pigmentation, tyrosinase and melanin biosynthesis are over-represented (Figure S2, Supporting Information).

Table 3.2 DEGs found between tissues and treated or non-treated individuals.

	TV	**TG**	**TD**
TM	190	3070	0
TV	-	1485	86
TG	-	-	2366
CG	-	5433	-
CV	1784	-	-

T stands for treated, C for non-treated (control), V for ventral caudal ray (sword), M for middle caudal ray, D for dorsal caudal ray

DEGs in the developing gonopodium

Using the same comparison-scheme as for the sword, we determined DEGs in the development of the gonopodium. Expressed genes in gonopodium tissues (TG and CG, see Figure 3.1) between treatment and control fish were compared. A total of 5,433 genes were found to be differentially expressed: 2,789 genes of those were up-regulated and 2,644 were down-regulated in TG compared to the non-maturing control fish (CG) (Table S4, Supporting Information). The GO term enrichment analysis of those 5,433 genes revealed that 240 functional categories are significantly over-represented compared to the assembled transcriptome (Table S5, Supporting Information). Similar to our analyses on the sword (TV), we conducted further analyses restricted to the gonopodium-specific DEGs. We found 816 up-regulated and 528 down-regulated genes in TG compared to CG, TM and TD (Figures 3.1, 3.2, Table S6, Supporting Information). In the gonopodium-specific DEGs, we found 35 overrepresented GO terms compared to reference transcriptome (Table S7, Supporting

Information).

Gene expression patterns shared between the sword and gonopodium

To identify the common molecular mechanisms that are found during both sword and gonopodium development, we compared the lists of DEGs and enriched GO terms from the comparisons of 'TV and CV' and 'TG and CG'. We found that 643 up-regulated and 610 down-regulated genes are shared (Table S8, Supporting Information). Independent GO terms enrichment analyses showed that 132 of enriched terms are common while overrepresented GO terms for only in sword and gonopodium are 240 and 108, respectively (Table S9, Supporting Information).

Gene expression differences in the developing anal and ventral caudal fins

To identify which genes were initially differentially expressed in untreated (control) ventral caudal (CV) and gonopodium (CG) (control anal fins) before the sword and gonopodium visibly developed, we compared the expression patterns between those two tissues solely in control fish. 115 of the DEGs were up-regulated in the control ventral caudal fin (CV) while 39 of the DEGs were up-regulated in the control gonopodium (anal fin) (CG) (Table S10, Supporting Information). We further compared those DEGs (CV and CG) to ones found in the developing sword (TV and CV) to test if the initial difference of the expressed genes due to tissue (ventral caudal and anal fin) specificity is increased, decreased, or completely lost during sword and gonopodium development in the treated fish. We found that 27 genes out of 115 up-regulated DEGs in CV were further up-regulated during the development of the sword (TV and CV). Those interesting genes include eight collagen genes (*col1a1*, *col1a2*, *col5a2*, *col5a3*, *col10a1*, *col12a1*, *col14a1*, *col27a1*), one *Hox* gene (*hoxc13*) and *insulin-like growth factor 2* (*igf2*) (Table S10, Supporting Information). In the control (not treated with testosterone) anal fin (CG), 12 out of 39 up-regulated DEGs retained their gene expression differences in the developing gonopodia as well (TG and CG), including four *Hox* genes (*hoxa9*, *10*, *11*, and *lhx9*) (Table S10, Supporting Information). These findings suggest that pre-existing differential expression patterns between anal and ventral caudal fins (tissue-specific variation) during early ontogeny are partly further maintained when they became transformed into the male-specific organs.

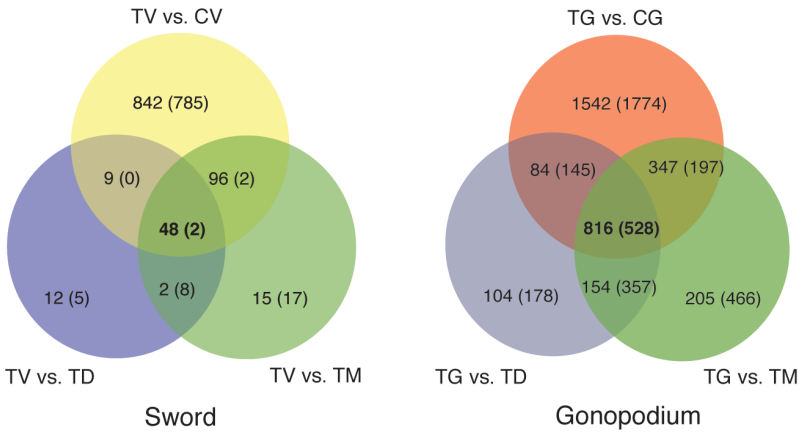

Figure 3.2 Venn diagram showing co-differentially expressed genes among different pair-wise comparisons for sword and gonopodium.

Numbers indicate the up-regulated genes and those in parentheses the down-regulated genes respectively. TV stands for treated ventral ray (sword), TD for treated dorsal ray, TM for treated middle ray, TG for treated anal fin ray (gonopodium), CV for non-treated ventral ray in control fish and CG for non-treated anal fin ray in control fish. * T stands for treated, C for non-treated (control), V for ventral caudal rays (sword), M for middle caudal rays, D for dorsal caudal rays, G for gonopodium (anal fin rays).

Table 3.3 Sword-specific DEGs from the limited gene set (TV and CV, TV and TD, TV and TM) between tissues and treatments.

ID	Gene name	Ensembl Gene id	Ensembl Gene description
		Up-regulated in developing sword	
CUFF.36200.1	AGTR1	ENSXMAG00000020155	angiotensin II receptor, type 1 [Source:HGNC Symbol;Acc:336]
CUFF.22492.1	ANGPTL5	ENSXMAG00000010840	angiopoietin-like 5 [Source:HGNC Symbol;Acc:19705]
CUFF.15255.1	ANO5 (1 of 2)	ENSXMAG00000007021	anoctamin 5 [Source:HGNC Symbol;Acc:27337]
CUFF.1503.1	ASIP	ENSXMAG00000012156	agouti signaling protein [Source:HGNC Symbol;Acc:745]
CUFF.38557.1	BCAN (2 of 2)	ENSXMAG00000015085	brevican [Source:HGNC Symbol;Acc:23059]
CUFF.11483.1	CKAP4	ENSXMAG00000001319	cytoskeleton-associated protein 4 [Source:HGNC Symbol;Acc:16991]
CUFF.35437.1	COL10A1 (2 of 2)	ENSXMAG00000020162	collagen, type X, alpha 1 [Source:HGNC Symbol;Acc:2185]
CUFF.40385.1	DSTN	ENSXMAG00000006139	destrin (actin depolymerizing factor) [Source:HGNC Symbol;Acc:15750]
CUFF.24718.1	ECEL1 (1 of 2)	ENSXMAG00000002319	endothelin converting enzyme-like 1 [Source:HGNC Symbol;Acc:3147]
CUFF.37038.1	FKBP9	ENSXMAG00000003160	FK506 binding protein 9, 63 kDa [Source:HGNC Symbol;Acc:3725]
CUFF.7252.1	GFRA1 (1 of 2)	ENSXMAG00000014650	GDNF family receptor alpha 1 [Source:HGNC Symbol;Acc:4243]
CUFF.22162.1	GJA1	ENSXMAG00000005269	gap junction protein, alpha 1, 43kDa [Source:HGNC Symbol;Acc:4274]
CUFF.12280.1	INHBB (1 of 2)	ENSXMAG00000006435	inhibin, beta B [Source:HGNC Symbol;Acc:6067]
CUFF.12283.1	INHBB (1 of 2)	ENSXMAG00000006435	inhibin, beta B [Source:HGNC Symbol;Acc:6067]
CUFF.27942.1	KCNH8	ENSXMAG00000000864	potassium voltage-gated channel, subfamily H (eag-related), member 8 [Source:HGNC Symbol;Acc:18864]
CUFF.27947.1	KCNH8	ENSXMAG00000000864	potassium voltage-gated channel, subfamily H (eag-related), member 8 [Source:HGNC Symbol;Acc:18864]
CUFF.25856.1	LURAP1 (1 of 2)	ENSXMAG00000002255	leucine rich adaptor protein 1 [Source:HGNC Symbol;Acc:32327]
CUFF.17142.1	MMP20	ENSXMAG00000001558	matrixmetallopeptidase 20 [Source:HGNC Symbol;Acc:7167]
CUFF.26478.1	PANX3	ENSXMAG00000006184	pannexin 3 [Source:HGNC Symbol;Acc:20573]
CUFF.33889.1	PAX9	ENSXMAG00000015762	paired box 9 [Source:HGNC Symbol;Acc:8623]
CUFF.15331.1	PDIA4	ENSXMAG00000018874	protein disulfide isomerase family A, member 4 [Source:HGNC Symbol;Acc:30167]
CUFF.25172.1	PMEL (1 of 2)	ENSXMAG00000003314	premelanosome protein [Source:HGNC Symbol;Acc:10880]
CUFF.15589.1	PPIC	ENSXMAG00000015311	peptidylprolylisomerase C (cyclophilin C) [Source:HGNC Symbol;Acc:9256]
CUFF.25279.1	RCN1	ENSXMAG00000014239	reticulocalbin 1, EF-hand calcium binding domain [Source:HGNC Symbol;Acc:9934]
CUFF.11860.1	RCN3	ENSXMAG00000002579	reticulocalbin 3, EF-hand calcium binding domain [Source:HGNC Symbol;Acc:21145]

CUFF.25595.1	SLC13A5 (1 of 2)	ENSXMAG00000004065	solute carrier family 13 (sodium-dependent citrate transporter), member 5 [Source:HGNC Symbol;Acc:23089]
CUFF.5285.1	STAC2 (2 of 2)	ENSXMAG00000002187	SH3 and cysteine rich domain 2 [Source:HGNC Symbol;Acc:23990]
CUFF.26167.1	TBX3	ENSXMAG00000005372	T-box 3 [Source:HGNC Symbol;Acc:11602]
CUFF.36189.1	TENC1 (2 of 2)	ENSXMAG00000018693	tensin like C1 domain containing phosphatase (tensin 2) [Source:HGNC Symbol;Acc:19737]
CUFF.29119.1	TNC (1 of 2)	ENSXMAG00000004876	tenascin C [Source:HGNC Symbol;Acc:5318]
CUFF.28413.1	TNC (2 of 2)	ENSXMAG00000018617	tenascin C [Source:HGNC Symbol;Acc:5318]
CUFF.28417.1	TNC (2 of 2)	ENSXMAG00000018617	tenascin C [Source:HGNC Symbol;Acc:5318]
CUFF.23730.1	TRPC6 (1 of 2)	ENSXMAG00000010842	transient receptor potential cation channel, subfamily C, member 6 [Source:HGNC Symbol;Acc:12338]
CUFF.26841.1	TYR (1 of 2)	ENSXMAG00000011209	tyrosinase [Source:HGNC Symbol;Acc:12442]
CUFF.25560.1	TYRP1 (2 of 2)	ENSXMAG00000004910	tyrosinase-related protein 1 [Source:HGNC Symbol;Acc:12450]
CUFF.4663.1	XDH	ENSXMAG00000003461	xanthine dehydrogenase [Source:HGNC Symbol;Acc:12805]
CUFF.11637.1	#N/A	ENSXMAG00000005039	fibulin-7 isoform 1
CUFF.12274.1	#N/A	#N/A	#N/A
CUFF.17007.1	#N/A	ENSXMAG00000018184	A fish specific (TGD) ortholog of hedgehog interacting protein like 1
CUFF.22535.1	#N/A	ENSXMAG00000011006	neurexin 2a
CUFF.23770.1	#N/A	#N/A	nilt2 leucocyte receptor
CUFF.31467.1	#N/A	ENSXMAG00000015953	tubulin alpha
CUFF.31478.1	#N/A	ENSXMAG00000015953	tubulin alpha-1a chain
CUFF.33581.1	#N/A	ENSXMAG00000003979	ox-2 membrane glycol
CUFF.4179.1	#N/A	#N/A	gdnf family receptor alpha-1-like
CUFF.7419.1	#N/A	ENSXMAG00000000509	Connexin
CUFF.8191.1	#N/A	ENSXMAG00000006973	spondin-1 precursor
CUFF.8192.1	#N/A	ENSXMAG00000006973	spondin-1 precursor
		Down-regulated in developing sword	
CUFF.35587.1	#N/A	#N/A	pro-neuregulin- membrane-bound isoform
CUFF.12776.1	#N/A	#N/A	#N/A

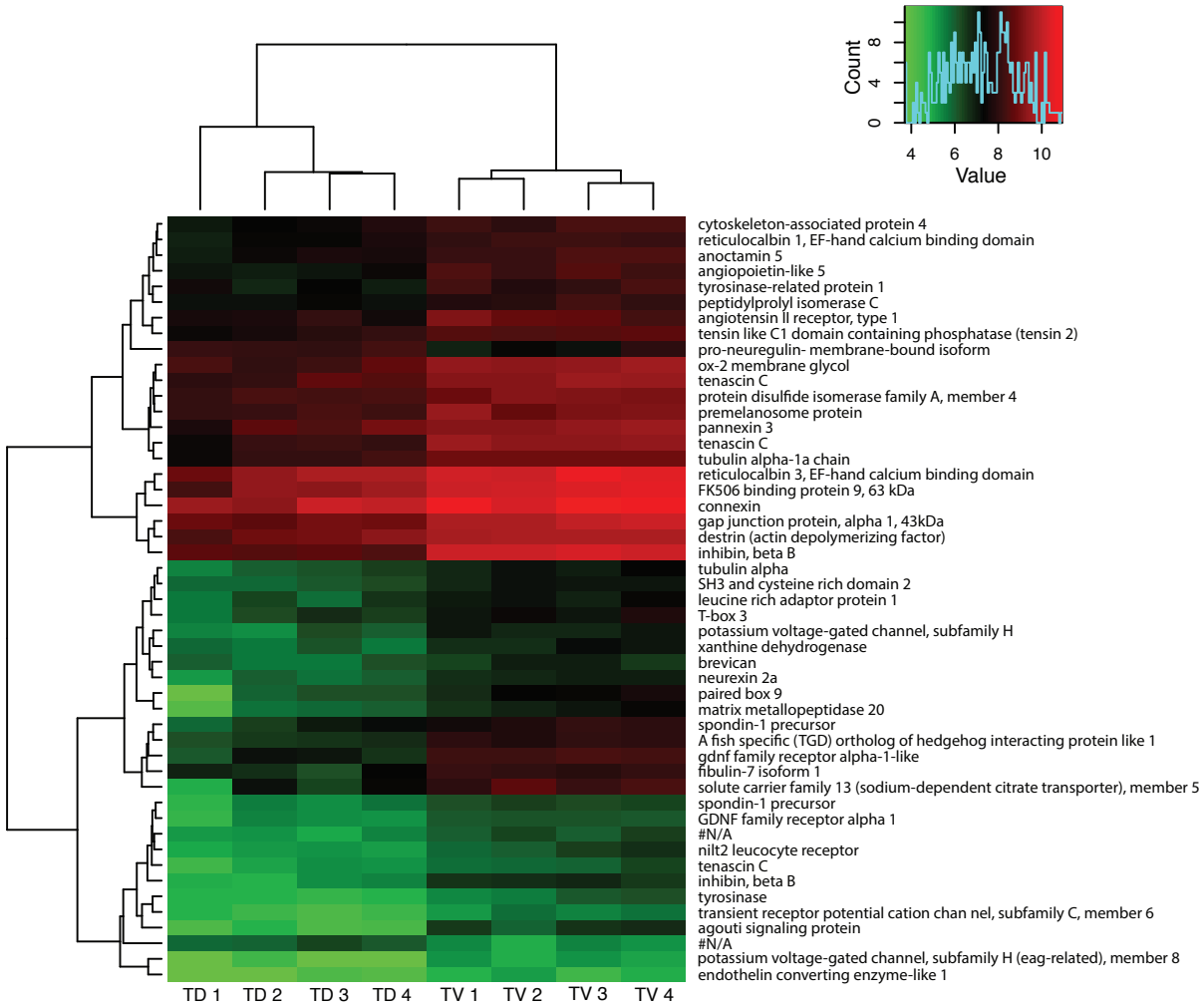

Figure 3.3 Sword specific-gene expression.

Heatmap of the limited gene set for sword displaying significantly differential expression between treated dorsal (TD) and treated ventral (TV, sword) rays. Color coding represents normalized expression data (variance stabilization transformed data implemented in DESeq). * T stands for treated, V for ventral caudal ray (sword), D for dorsal caudal ray. One locus with extreme values was excluded (COL10A1: CUFF. 35437.1).

Discussion

This study provides the first comprehensive catalogue of the genes activated in two developing male-specific traits, the gonopodium and the sword. Our analyses identified hundreds of genes linked to the development of these two evolutionary novelties. That so many genes show expression differences surprised us initially, but might reflect the complexity of the biological processes involved in the transformation of a simple fin into a sperm transfer organ or a colorful exaggerated male ornament. Genes activated (positively or negatively) in the developing tissues can be: 1) hormone responsive genes involved in the initiation and continuation of the development of traits during sexual maturation, 2) genes regulating cellular processes during post-embryonic development and 3) genes contributing to downstream morphological changes such as cell proliferation, outgrowth of fin rays, addition of new segments and coloration. We find many genes already known to be involved in those significant biological functions and numerous male biased or sex-specific genes as well.

Effects of sex on testosterone response would be trivial in this experiment. It is not feasible to determine sex of juveniles before sexual maturation since gonad development and sexual differentiation have not yet occurred at the age of three months. The amount of testosterone in this experiment would be sufficient to eliminate the original sex differences. If putative male or female juveniles responded differentially to the hormone, one might expect also that the hormone-induced sword and gonopodium should differ between the sexes. However, all hormone induced swords and gonopodia developed very similarly in terms of their morphology and timing of development. Furthermore, we observed that all induced swords and gonopodia are morphologically almost identical among juveniles during the extended duration of testosterone treatment at 18 days (induced sword and gonopodium development is completed at 18 days of treatment) (Offen *et al*. 2008, 2009). However, the possibility of functional sex reversal by exogenous steroid hormone cannot be completely ruled out since this phenomenon is not rare in teleost fishes (Pandian & Sheela, 1995). If sex hormones exclusively determined the sex and sexual differentiation in this fish species, hormone treated fish in our experiments would be expected to be all males.

Genes for hormone response: the androgen signaling pathways

The differential expression of hormone responsive genes such as androgen and/or estrogen would be expected in testosterone-induced swords and gonopodia in juveniles. Sex hormones

play an important role in the development of secondary sexual traits. Artificially induced gonopodia by exogenous testosterone in juveniles, even in females of other poeciliid fish such as *Gambusia affinis*, suggests that androgen signaling is involved in the development of the gonopodium. Therefore, androgen receptors would be the first genes to be considered as key genes to orchestrate the complete network leading to the differential expression of all other genes in the development of both traits. However, differential expression of androgen receptors were not found between the developing sword (TV) and untreated control fin (CV). But, differentially expressed androgen induced 1 (AIG-1) and many other down-stream targets of androgen signaling pathways such as *fgf7* and *fgf16* in the developing sword (TV) were found (Table S1, Supporting Information) and might indicate an activation of androgen signaling. Similarly, differential expression of the androgen receptor gene was not observed between the developing gonopodium (TG) and untreated control anal fin (CG) (Table S4, Supporting Information). This could be a sign of no expression differences of androgen receptors in the developing gonopodium or, alternatively, indicate on equal expression of androgen receptor in both tissues. The latter is more likely since our previous study using in situ hybridization (Offen *et al.* 2013) detected the expression of androgen receptor β (*arβ*) in the untreated control anal fin. This hypothesis is further supported by our transcriptome data demonstrating that differential expression of androgen receptors between the anal fin (CG) and the ventral caudal fin (CV) in untreated control fish (Table S10, Supporting Information). It suggests that the regulation of androgen receptor originally differs between those two tissues before the development of the sword and the gonopodium. In situ hybridization showed that up-regulation of androgen receptor β (*arβ*) was detected constantly in both developing gonopodia and untreated control fins during development whereas swords showed a more dynamic and complicated expression patterns of *arβ*, e.g., its expression depends upon timing and the position of rays. Taken all information into consideration it seems likely that androgen signaling is differentially regulated in the developing sword and gonopodium. The dynamic nature of *arβ* expression in the sword could explain why sword develops from the ventral caudal fin only.

Male biased, sex-differentiation, sex-determination genes

On a related note, the differential expression of male-biased, sex-differentiation or sex-determination genes is expected since these traits are male-specific and developed during sexual maturation in natural condition. We have indeed found several genes that were

previously identified as having sex-specific gene expression patterns. For example, *cyps* and *gpx 7, 8* (Tables S4 and S8, Supporting Information) were differentially expressed in the developing sword and/or gonopodium. *Cyps* plays a role in catalyzing the oxidation of organic substances and *gpx 7*, *8* are antioxidant genes (Zheng *et al.* 2013). The sword-specific gene list (Table 3.3) shows several sex-specific development related genes such as *col10a1* and *inhbb*. *Inhbb* has been suggested to regulate the sexually dimorphic differentiation of gonads. It contributes to the formation of the coelomic vessel, which is critical for testis development while it is suppressed by *Wnt4* in the ovary (Yao *et al.* 2006). *Wnt4* – a well-known gene for its role in female sex development (Chassot *et al.* 2012; Li *et al.* 2014) - was down-regulated in both the sword and the gonopodium and it has previously been suggested to be a female sex determination gene (Forconi *et al.* 2013). It appears that female-biased genes are down-regulated or inhibited. Another interesting gene is *Sox9*, which has been identified as a sex-determination gene (Sekido & Lovell-Badge 2008). Its conserved role during gonad development in vertebrates is known (Yokoi *et al.* 2002) and it is also associated with testis differentiation in mouse (Wainwright *et al.* 2013) and other mammals (Barrionuevo *et al.* 2012). In our study, several *sox* genes are differentially expressed in the sword (*sox5*, and *10* are up-regulated- while *sox2*, *3*, *9* are down-regulated) (Table S1, Supporting Information) and in the gonopodium (*sox4*, *6*, *18* up-regulated while *sox3*, *9*, *13* down-regulated) (Table S4, Supporting Information).

However, regarding the potential role of these genes as master regulators for sword and gonopodium development or sexual differentiation it should be noted that the developmental processes of hormone-induced sword/gonopodium might still differ from those of naturally developed ones. So far, too little is known about sex-determination and differentiation in *Xiphophorus*. In general, sexual differentiation could be regulated by sex chromosomes in a cell-autonomous fashion or by a sex-specific hormone signal received from the gonads or other tissues (Bear & Monteiro 2013). It is likely that sex biased and/or sex-determination genes are involved in the development of hormone-induced sword/gonopodium. Sex-biased or sex-specific genes that were identified in this study should be the focus of more future attention in an effort to investigate whether they are involved in the origin of sexual dimorphic traits in *Xiphophorus hellerii*.

Shared genes between the development of the sword and the gonopodium: signals of co-option

Commonly expressed genes during sword and gonopodium development offer intriguing insight into the potentially shared genetic mechanisms underlying both of these two types of male specific traits. Previous studies already suggested that genes expressed in the evolutionarily 'older' trait, gonopodium, might be co-opted during the ontogeny and evolution of the sword, a more recent evolutionary innovation in the genus *Xiphophorus* (Zauner *et al.* 2003; Offen *et al.* 2008). Indeed, our transcriptome analysis supports this idea as good portions of genes (about 70% of DEGs) are shared between the sword (TV and CV) and gonopodium (TG and CG) (see Tables S8 and S9 for the full list, Supporting Information). Further investigation is required for a deeper understanding of these potentially common genetic regulatory pathways and the role that they play in each of the gonopodium and sword developmental processes.

Our data also suggest that the development of both traits in swordtails seems to be a result of pleiotropic effects of several genes and/or some that are co-opted from many embryonic developmental genes. Shared genes by both male-specific traits are involved in many developmental processes (e.g., tissue development, skeletal system development, collagen fibril organization, system development and various organ development, etc.) and morphogenesis (e.g., organ, anatomical and skeletal system) related biological functions (see full list, Table S9, Supporting Information). This list includes many of those pathways that are involved in embryonic developmental gene networks (i.e., limb development, organ development). The recruitment of already existing gene networks using "gene network co-option" has been suggested to be a usual way for the development and evolution of novel traits (Fraser *et al.* 2009; Monteiro & Podlaha 2009). Recently, the co-option of a gene network has been suggested to underlie the origin of a novel trait – for example, red patches of pigmentation on butterfly wings may have resulted from the co-option of eye-developmental genes (i.e., *optix*) (Monteiro 2012). Such cases are also observed in the development of morphologically specific organs or sexually selected traits in other animal groups (Moczek & Rose 2009). For example, *Hox* genes are well-known principal transcriptional regulators of animal body regionalization in embryonic development (Meyer & Málaga-Trillo 1999; Kopp 2011; Tanaka *et al.* 2011). *Hox* genes have also been suggested to be key-players in the development and evolution of novel complex traits such as beetle horns (Wasik *et al.* 2010), male genitalia (i.e., imaginal discs) (Estrada *et al.* 2003), and a

secondary sexual trait - sex combs in *Drosophila* species (Barmina & Kopp 2007). We also found many *hox* genes (i.e., *dlx*, *lhx9*, *satb2*, *zhx2* and etc.) to be commonly expressed in both evolutionary novelties under consideration here, the sword and the gonopodium. Taken together, it is likely that genetic regulatory networks were co-opted during gonopodium development and evolution and were subsequently deployed as well in the later evolution of another novelty, of the sword.

Genes contributing to downstream morphological changes

a) Gene expression in the sword

A large number of genes were found that appear to be involved in the development of the sword. In the comparison between TV and CV, we found many genes and related functions that might be expected from the complicated morphological and biological changes occurring during its development. Genes or pathways involved in early embryonic development are activated again during the metamorphosis of fin rays into the sword. The analyses revealed various embryonic organ development-related functions such as chordate embryonic development, embryonic morphogenesis, embryonic organ development, embryonic skeletal system development, and in utero embryonic development. Among the sword-specific genes (Table 3.3), several genes with crucial roles during embryonic development and organogenesis such as *pax9* and *tbx3* are found (Table 3.3). For instance, *Pax9* is known to play, pleiotropically essential roles in the development of the craniofacial skeleton, the dentition (Peters *et al.* 1999) and tooth morphogenesis in mice (Kapadia *et al.* 2007). *Tbx3* is also thought to play a role in the posterior/anterior axis of tetrapod forelimb (Gibson-Brown *et al.* 1996), heart development (Ribeiro *et al.* 2007), and genital development (Ballim *et al.* 2012). However, a function for them in the development of the sword had not been suggested before. This indicates that even though the sword development happens at a post-embryonic stage, it re-employs the same genetic toolkit used for many fundamental processes during early embryonic development.

Many genes (described by their respective GO terms) that are expected to be responsible for morphological changes of the developing sword are indeed activated (Table S2, Supporting Information). The development of the sword includes various morphological changes such as outgrowth of fin rays and addition of segmentation. It should also be noted that all these genes and functions are simultaneously activated at early stages of the sword's development even before all characteristics of the sword such as the elongation of fin rays, coloration and segmentation have appeared.

b) Why does only the ventral caudal fin develop into sword?

Dorsal and ventral parts of the caudal fin are morphologically very similar (e.g., number of rays and segments) before the sword develops. Yet, only the ventral caudal fin rays undergo a transformation into a sword during sexual maturation. Interestingly, through testosterone-treatment also the dorsal caudal fin rays are occasionally induced to produce a sword-like protrusion, although it does not develop into a fully developed sword even with prolonged exposure to testosterone (Eibner *et al.* 2008). Therefore, DEGs in the comparison between the ventral and dorsal caudal fins (TV and TD) might inform which genes are functionally necessary for the origination of the fully developed sword. We found 71 up-regulated and 15 down-regulated genes in the ventral caudal fin (sword) compared to dorsal portion of the caudal fin (Table S11, Supporting Information). Most of the up-regulated genes are the same as those in the restricted gene list (Table 3.3). However, several genes such as *col10a1, fndc7*, *clec19a*, *anionic trypsin-2-like* (collagen catabolic process)*, zona pellucida-like domain-containing protein 1-like* are also overexpressed in the ventral caudal fin compared to the dorsal portion of the caudal fin (Table S11, Supporting Information). Those genes are generally known to be involved in cell proliferation and growth. This could be simply explained by the fact that the ventral caudal fin is growing faster compared to the dorsal fin. However, it seems worthwhile noting that compared to other tissue comparisons (e.g., TD vs. CV, TV vs. CV), a rather smaller number of genes are differently expressed between the dorsal (TD) and the ventral caudal fin (TV) in the testosterone-treated fish (Table 3.2), probably indicating their similarities in their "ontogenetic potential". Gene expression of those genes might contribute to the transformation of the ventral rays into the sword, although it is not clear how far "upstream" these genes are in the "command-chain" permitting the sword transformation or even inducing it. Testosterone treatment induces only a small protrusion in the dorsal rays of the caudal fin in *X. hellerii* (Sangster 1948; Dzwillo 1964; Offen *et al.* 2008) and we found earlier, that the transplantation of a single ventral sword ray to the dorsal caudal fin induced an ectopic sword dorsally (Eibner *et al.* 2008). Apparently the sword signal is carried with the transplanted ventral ray to new dorsal caudal fin location. Therefore, our results suggest that rather small changes of gene expression in the ventral caudal fin rays lead to the different fate of dorsal and ventral caudal fin rays during sexual maturation and the transformation of simple fin rays, into structures that as a composite make up the sword.

Color genes in the sword (The colorful sword)

The sword is a composite trait consisting of a yellow coloration and a black ventral margin with remarkable inter- and intra-specific variation of coloration (Basolo 1991; Meyer 1997). Gene lists from other comparisons provide further detailed information of color gene expression in the developing sword. Several up-regulated melanogenesis-related genes (the regulation of melanocyte-related pigmentation) such as *asip*, *pmel*, *tyr*, *mc1r* and *mlph* in the developing sword (Table S1, Supporting Information) would be responsible for black coloration. Genes involved in expressing yellow or orange coloration are also up-regulated in the developing sword. For example, *xdh* is known to regulate the synthesis of pigments for yellow or orange coloration found in xanthophores (Oliphant & Hudon 1993).

Interestingly, in our study the color genes show slightly different expression patterns based on the different comparisons. For example, the expression of *xdh* and *asip* seems to be sword-specific since they are up-regulated in the developing sword (TV) compared to the treated caudal fins (TM and TD) and untreated caudal fins (CV) (Table 3.3). No differential expression between the middle caudal fin (TM) in treated fish and the ventral caudal fin (CV) in control fish further support the sword-specific gene expression of *xdh* and *asip*. On the other hand, *mc1r* shows differential expression only in a comparison between the sword (TV) and non-treated ventral caudal fin (CV) (Table S1, Supporting Information).

Other yellow coloration carotenoid related color genes [i.e., *bco2*] seem to be more influenced by a hormonal response since they are up-regulated in the treated middle caudal fin (TM) compared to untreated ventral caudal fin (CV). This finding indicates that some color genes (i.e., *bco2*) are testosterone hormone-induced genes, while others (e.g., *xdh* and *asip*) are more tissue or sword-specific genes. In short, our data show that several color genes are expressed during early sword development. These results suggest that genes down- or upstream of color genes should be carefully considered to understand the whole process of the transformation of the ventral rays of the caudal fin into a sword.

c) Gonopodium gene expression

As in the sword, active genes with functions such as responses to hormone stimuli, embryonic organ development, and organ morphogenesis are also expressed in the developing gonopodium. Interestingly, our results show that the number of DEGs in the developing gonopodium is approximately three times greater than that in the developing sword. Obviously, the gonopodium is a more complex structure than the sword and hence,

this result would make sense since the size of genetic networks can be translated into more diverse morphological structures as, for example, much fewer genes are involved in hair coloration than more complex traits such as craniofacial shape in humans (Claes *et al.* 2014). However, the earlier onset of the gonopodium development relative to the sword development might simply add to the observed result that more genes are being activated in the developing gonopodium than in the sword.

In addition to a larger number of genes expressed in gonopodium, there are several unique characteristics in its gene expressions, compared to the sword (Table S9, Supporting Information). For example, GO terms for dorsal/ventral pattern formation and regionalization were found only in the gonopodium, but not in the sword (Table S9, Supporting Information). The gonopodium develops a very specific proximal-distal (PD) axis difference due to its specialized distal structures such as hooks and a claw (Figure S3, Supporting Information). Although in the early gonopodium those traits are not developed yet, the specific pattern gene expression might determine cell fate in the early developmental stages. Activation of the *Wnt*-signaling pathway is a well-known biological pathway that regulates PD axis formation. We found four *wnt* genes (*wnt4*, *7a*, *9b* and *11*) to be down-regulated in the developing gonopodium (TG) compared to anal fin in untreated fish (CG) and particularly the *wnt7* down-regulation compared to other tissues in the treated fish might be worth following up on as wnt-genes are often implicated to play a role in the development of complex features and adaptive structural differences such as the shape of the neurocranium in fish (Parson *et al.* 2014) and tetrapods (Claes *et al.* 2014).

If those genes were activated in anal fin in untreated fish (CG), we would expect to detect a down-regulated expression in the developing gonopodium (TG) in treated fish. Alternatively, the expression of those genes could be suppressed in male traits induced by testosterone treatment since, for example, *wnt4* genes are known to be one of the female sex determination genes in fish (Forconi *et al.* 2013). Homologues of *fgf9* and *sry* - known to regulate mouse testis anterior and posterior determination (Hiramatsu *et al.* 2010) - are also up-regulated in the developing gonopodium (Table S4, Supporting Information). In future studies, investigations of gene expression patterns in the developing gonopodium and sword throughout different ontogenetic stages would allow for differentiating gonopodium-specific genetic networks from sword. And, once technically feasible, knock-down or - out experiments might help to establish functional roles more clearly (Kratochwil & Meyer 2015 a, b).

Are common sets of genes differentially expressed in male exaggerated ornaments?

Like swordtails, many other animal taxa have developed male exaggerated ornaments (or weapons) that are known to play a role in inter- and intersexual selection. Considering the sword gene list, we were interested in whether there are shared or conserved genetic pathways between the sword and other sexually selected male ornaments. Interestingly, several genes in the sword gene list are expressed or involved during development of other male exaggerate ornaments (weapon) or sexually selected traits.

For instance, *fork head* (*fkh*) genes (e.g., *foxs*) are involved in sex combs as natural targets of *scr* in fruit flies (Ryoo & Mann 1999), *crol* and *cdc2* in eye span in stalk-eyed flies (Baker *et al.* 2009), *dll* or *dlx* in sex comb in *Drosophila* and butterfly eyespot size (Dworkin 2005; Monteiro *et al.* 2013), *bmp2* in comb mass in chickens (Johnsson *et al.* 2012), and *IGF* pathways and *InRs* in the rhinoceros beetle horns (Emlen *et al.* 2012; Lavine *et al.* 2013). These genes or its homologs such as several *fork head box* genes (i.e., *foxd3*), zinc finger proteins as the homologs of *crol, cdcs* (e.g., *cdc7*, *16*, *20*, *27* and *34*), *dlxs* (*dlx1*, *2* and *4*), *bmps* (*bmp1*), and *IGF* pathway related genes (*igf2*, *igfbp1, 2*, *4*, *6,* and *irs2*) were found to be expressed in the developing sword and gonopodium (Tables S1 and S4, Supporting Information). Nevertheless, we can not rule out the possibility that the common set of these genes simply function as a morphogens and/or growth factors for the morphological changes ensuring in the developing sword and gonopodium. However, for instance, it was shown that the *IGF* pathway might be responsible for exaggeration of the structure and by-product of the growth mechanism in beetle horn (Emlen *et al.* 2012). The fact that our identified candidate genes belong to the same gene families and also play a role in male exaggerated ornaments might suggest their potential role in different sexually selected traits across different animal lineages. This hypothesis clearly needs further study, but is an exciting finding that deserves follow-up work.

Conclusions

Comparative RNA-sequencing of evolutionary novelties such as the gonopodium and the sword provided insights into the set of genes that are involved in the development of these male specific traits. A large number of expression-biased genes were identified in this study: 5,433 and 1,784 of DEGs for the gonopodium and the sword, respectively highlighting the massive changes that are taking place during the transformation of the anal fin into a

gonopodium during the sexual maturation. The genes identified here and the mode of regulatory evolution suggested by our results represent the basis for further research on understanding the molecular mechanisms underlying the development of male specific traits in swordtails, in other male specific traits and even in sexually selected traits in animals generally.

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Data accessibility

The short read DNA sequences for this study have been deposited in the European Nucleotide Archive (ENA) under the accession code PRJEB8012.

Chapter 4. The Genetic Basis of Sexually Selected Traits Across Different Animal Lineages: Are the Genetic Mechanisms in Common?

(Manuscript in preparation)

Review Article

The Genetic Basis of Sexually Selected Traits Across Different Animal Lineages: Are the Genetic Mechanisms in Common?

Ji Hyoun Kang[1,2] and Axel Meyer[1,2*]

[1]*Lehrstuhl für Zoologie und Evolutionsbiologie, Department of Biology, University of Konstanz, Universitätsstraße 10, 78457 Konstanz, Germany*
[2]*Konstanz Research School Chemical Biology, University of Konstanz, Konstanz, Germany*

*Correspondence: Axel Meyer; Lehrstuhl für Zoologie und Evolutionsbiologie, Department of Biology, University of Konstanz, Universitätsstrasse 10, 78457 Konstanz, Germany; +49 (0)7531 88 4163; axel.meyer@uni-konstanz.de

Abstract

Sexual selection involving female choice or female preference ('inter-sexual' selection) or male-male competition ('intra-sexual' selection) is one of the key mechanisms for evolutionary diversification and speciation. In particular, sexual selection is recently suggested to be an important mode to drive the evolution of "novel" phenotype (i.e. "evolutionary novelty"). Despite extensive studies performed on sexually selected traits or male-specific ornaments (or weapon-like structures) with respect to their evolutionary origin and history and fitness benefits, relatively little is known about the molecular genetic mechanisms underlying their development. However, with emerging advances of genomic technologies (including whole transcriptome analysis using Next Generation Sequencing [NGS] techniques; RNA-Seq), some recent progresses have been made to understand the genetic background underpinning diverse sexually selected traits in different animal taxa. In the present review, we compile empirical data on the genes, genetic mechanisms or regulatory pathways underlying various sexually selected traits to explore whether the "common" genetic architectures shape the development and evolution of those traits across evolutionarily distant animal lineages. We show that the recruitment of pre-existing genetic network for a new purpose (i.e. gene network "co-option") is rather widespread in the development and evolution of sexually selected traits, indicating that particular genes or gene-sets are repeatedly involved in different sexually selected traits. Information of genes or genetic mechanisms regulating the development of sexually selected traits would be an essential piece to complete a whole picture of the origin and evolution of those traits.

Main Text

1. Introduction

Exaggerated male ornaments or weapon-like structures in various animal taxa, such as peacock's tails and beetle horns, grasp peoples' attention because of their extravagant appearance (coloration or morphology). Ever since Darwin described on sexual selection, biologists have much been fascinated in the origin and evolution of exaggerated male ornaments since they are thought to be subject to a "special" type of evolutionary processes. These male-specific traits are believed to be sexually selected, meaning that they are beneficial for mating success by attracting mates or winning over rivals, although the exaggerated traits can be costly and have possibly detrimental effects on the fitness due to the action of natural selection (e.g., vulnerability to predators; see Endler 1980). Instead of rather a simple explanation of this trade-off between sexual and natural selection, the evolution of sexually selected traits is suggested to be much more complex and has thereby become an interesting topic in evolutionary biology.

In general, two mechanisms are known to drive the evolution of sexually selected traits: inter-sexual selection (female choice or preference) and intra-sexual selection (male-male competition) (Andersson & Simmons 2006). Exaggerated male secondary sexual characteristics are often the evolutionary outcomes of female preference to choose their mates (Collins & Luddem 2002). Darwin hypothesized that female choice (or preference) leads to the evolution of male sexual ornaments (Darwin 1871) and many studies have provided empirical evidence supporting this idea [e.g., male tail (Moller 1994), extreme tail length in a widowbird (Pryke *et al*. 2001)]. Moreover, variation in female choice strengthens sexual selection for male ornaments in lark bunting (Chaine and Lyon 2008). The elaborated male ornaments might be used by females as a signal of male quality for choosing mates (Hamilton and Zuk 1982) in many animal taxa including birds, flies, beetles, fish, ungulates, and curstaceans (Berglund *et al*. 1996; Warren *et al*. 2013). Sizes of male ornaments often matter on sexual selection in several species. For example, wing patch size is the target of female choice in collared flycatchers (de Heij *et al*. 2011). It was also shown in barn swallow that male ornament size acts as a "honest" signal by reflecting their offspring longevity (Moller 1994). Stalk-eyed fly showed a positive association of male reproductive

morphology with enhanced fertility (Rogers *et al*. 2008). Length and darkness in lion's mane are signals for male qualities such as nutrition and fighting success (West and Packer 2002).

Some of exaggerated male traits such as claws in fiddler crabs (Clements *et al*. 2010; Dennenmoser and Christy 2013), horns of giant rhinoceros beetles (Emlen *et al*. 2007) and antler in red deer (Clutton-Brock & Albon 1979) are used as a weapon to deter rivals for mating competition. Exaggerated sexually-selected traits are usually associated with individual variation in the traits, based on age, size, nutritional condition and genotype since their expressions are highly condition-dependent (Warren *et al*. 2013).

The notion that male exaggerated ornaments are considered "evolutionary novelties" has brought interesting questions for their origin and evolutionary history including lineage specific loss and gain (Emlen *et al*. 2007). Evolutionary novelties or novel traits are generally described as structures or characters that are not homologous to any structures existed in ancestral lineages or any structures of the same species (Müller and Wagner 1991). Novel functional capabilities (e.g., flight, vision) or novel structural elements (e.g., hair and horn in mammals, scales in reptiles) are two categories of evolutionary novelties when the developmental origin of novel body parts considered (Wagner & Lynch 2010). Sexually selected traits are suggested to be evolutionary novelties in several cases of phylogenetic studies. Various beetle horns used to combat male rivals are considered a novel structure because they are neither found in relatives of other beetle groups nor found in a common ancestor in all beetles' lineages. The clade of squamate reptiles (lizards and snakes) shows the multiple origins of astonishing novel traits such as viviparity, snake-like lime, reduced body and venom (Sites *et al*. 2011).

Despite extensive studies performed on sexually selected traits in terms of their evolutionary origins and history, and fitness benefits, little is known about molecular mechanisms underlying the development of exaggerated male ornaments and sexually selected traits. An understanding of their developmental processes is essential to identify the origin of sexually selected traits (secondary sex specific traits) (Williams & Carroll 2009). In particular, unique developmental and evolutionary identities can be better understood by an investigation of the origin and divergence of the novel gene regulatory networks contributing to the morphological innovations (Wagner & Lynch 2010). If we know the genetic mechanisms of the origin and evolution of exaggerated male traits, then it will also help to understand the genetic mechanisms underlying evolutionary novelties. Information of genes or genetic mechanisms regulating the development of sexually selected trait (male

exaggerated ornaments) would be an essential piece to complete a whole picture of the origin and evolution of those traits.

While many studies have focused on the determination of genetic variation responsible for sexually selected traits (reviewed in Chenoweth and McGuigan 2010), research efforts on the identification of the 'causal' or associated genes or their regulations underlying the traits are much limited. Recently, however, more and more studies about genes or genetic pathways underlying sexually selected traits have been performed in various distantly related animal lineages [e.g., sword in swordtails (Kang *et al*. 2015), horn in beetle species (Kijimoto *et al*. 2009; Choi *et al.* 2010; Warren *et al.* 2014), antler in deer (Yao *et al*. 2012a, b; Zhao *et al*. 2013), plumage coloration in birds (Ekblom *et al*. 2012), and eye span in stalk-eyed flies (Warren & Smith 2007; Wilkinson *et al*. 2013). Recent advances of genomic technologies (including whole transcriptome analyses using Next Generation Sequencing [NGS] techniques) have facilitated researches in this field at the genome-wide level. However, few attempts have been made to compare the genetic mechanisms or genetic backgrounds of sexually selected traits among different animal taxa. With the accumulating information, we are now able to compare genes and genetic pathways among different sexually selected traits across different taxa in order to test whether common (or similar or completely different) genetic mechanisms contribute to the development of those traits. If they share the common or similar genetic mechanisms, then it will provide the basis of knowledge that different sexually selected traits evolve through the common molecular mechanisms. In the present review, we focus on the specific genes or genetic pathways involved in sexually selected traits, using recent emerging empirical data with NGS. The goals of this review are two-fold: (1) to summarize empirical data on the genetic mechanisms underlying sexually selected traits and (2) to explore whether the common (or shared) genetic mechanisms shape the development or evolution of the sexually selected traits across evolutionarily distant animal lineages. Although it is difficult to identify the target (or causal) genes or their genetic regulations underlying the traits by comparing expression genetic data, by doing so we aim to suggest a framework or guide for future research to look deeper into the genetic basis underlying the origin and evolution of the traits. It would allow us to get a starting piece to understand the complex of the conserved genetic mechanisms of the evolutionary novelties.

2. The genetic basis of various sexually selected traits (SST) or exaggerated sexual ornaments in animal taxa

Various sexually selected traits or sex-specific ornaments can frequently be observed in nature from diverse animal groups such as insects, fishes, birds and mammals. Examples include the horns in beetles (Emlen 2001), eye-span in stalk-eyed flies (Baker *et al.* 2009), sex combs in fruit flies (Kopp 2011), plumage coloration in birds (Scordato *et al*. 2012) and antler in deer (Vanpe *et al*. 2007). Despite accumulating evidence of their crucial ecological role, little is known about the genes or genetic pathways underlying the traits, retarding the progress of our understanding of the developmental origin of those evolutionary novelties. Recently, however, some progresses have been made to identify a handful of (candidate) genes or genetic network that might account for sexually selected traits. Here, we list several sexually selected traits that have been studied on their genetic mechanisms or genetic pathways (Table 4.1).

2.1. Sexually selected traits on flies

Development and evolution of sexually selected male ornaments in insects have previously been reviewed (Emlen & Nijhout 2000). The most intensive investigations on the gene expression or genetic mechanisms of sex specific or sexually selected traits have been performed for fly species in insects. Stalk-eyed flies are well-studied model organisms to understand the genetic mechanisms underlying sexually selected male traits in a diverse group of species. Stalk-eyed fly species have evolved male exaggerated hypercephaly, known as “eyestalks”, which are the lateral projections of the head capsule (Warren & Smith 2007). Female diopsid flies use this trait as an indicator of male quality (Warren & Smith 2007). Male eye-span is known to be a sexually selected trait and eyestalk size shows large amount of interspecific variation. With respect to its genetic regulatory architecture, the expression of *hedgehog* (*hh*), *wingless* (*wg*), *engrailed* (*en*) and a transcription factor, *defective proventriculus* (*dve*) as their *Droshophila* homologues was found in eye-antennal disc in stalk-eyed flies (Hurley *et al*. 2001; Warren & Smith 2007) (Table 4.1). Using EST (Expressed Sequence Tag) sequencing and microarray analysis, a study of eye-antennal imaginal discs in stalk-eyed flies revealed several candidate genes such as *Crooked legs* and *cdc2* (Baker *et al*. 2009). Gene expression patterns in the developing tissues of the eyestalk indicated the potential role of gene duplication in the evolution of sex specific traits (Baker *et*

al. 2012). Microarray analysis has further shown that sex-biased gene expression is associated with the development of dimorphic eyestalk (Wilkinson *et al*. 2013).

Sex comb in males is another extensively studied, sexually dimorphic trait in *Drosophila* species although it was suggested not to directly related to sexual selection (Snook *et al*. 2013). This trait is used for males to grasp female abdomen and genitalia for their successful copulation. Several genes are found to be involved in the development of sex comb. *Dachshund* (*dac*), which is known to have a conserved function in sensory organ and appendage development in insects, is recently shown to be involved in the sex-comb development (Atallah *et al*. 2014). Candidate gene approaches identified *scr* and *dsx* (Devi & Shyamala 2013) that also contribute to the sex-comb development in *Drosophila* (Graze *et al*. 2007). Sex-determining genes such as *daschund* (*dac*) and homeobox genes have also been found to be expressed during its development (Barmina & Kopp 2007; Kopp 2011; Tanaka *et al*. 2011; Devi & Shyamala 2013; Atallah *et al*. 2014).

2.2. Exaggerated male traits in beetle horns

Recent seminal studies uncovered the novel genetic mechanisms underlying the rhinoceros beetle horns, sexually selected male weapon. Beetle horn has become one of the famous examples of male exaggerated ornaments because of its magnificent size relative to their body and extraordinarily high levels of its interspecific variation in terms of size and shape. It is a sexually selected trait through female choice and also used as a weapon for male-male competition. Using combined analyses on comparative phylogenetic studies of horn evolution with developmental investigations of horn growth (Emlen *et al*. 2005, 2007), Emlen *et al.* (2006) proposed the "evolutionarily labile horns" hypothesis that although the earliest (ancestral) horn morphologies have not been resolved in the scarab superfamily (Coleoptera: Scarabaeoidea) (which beetles with horns belong to), all modern phylogenies suggest that the gain and loss of horn are labile during evolutionary history and its form sometimes changes rapidly and dramatically (Emlen *et al*. 2006). They found that genetic changes on the domain such as *hh*, *wg* and *dpp* signals determine the precise locations of the horn outgrowth. Even subtle changes on the genes involved in the limb-patterning pathways can lead to drastic changes in horn forms and shapes (Emlen *et al*. 2006). The same research team published seminal studies on the genetic mechanisms on the beetle horn that revealed novel functions of the genes involved in the sexually selected trait in beetles. Insulin signaling pathways, a major regulator for tissue growth and body size (Froesch *et al*. 1985; Ohlsson *et al*. 1998), has been suggested as candidate genetic pathways for the evolution of the beetle 'horn'

(Emlen *et al*. 2006). Recently, they further showed significantly higher sensitivity of cells to insulin/Insulin-like Growth Factor (IGF) in beetle horn (weapon) compared to other traits (genitalia and wing) in rhinoceros beetle (Emlen *et al*. 2012). This increased cellular sensitivity to insulin/IGF pathways is suggested to cause the extreme growth because it acts a reliable signal of better male quality or it is simply by-product of the growth mechanisms (Emlen *et al*. 2012). Furthermore, *insulin receptors* (*InRs*) are found to be responsible for polymorphic horn developments in sexually dimorphic male horned beetles (Lavine *et al*. 2013).

2.3. Sword in swordtail fish

"Sword" in swordtail fish in the genus *Xiphophorus* is one of the well-known examples of sexually selected traits in fish. Some *Xiphophorus* species, swordtails, but not others, the platies, have a male-specific trait, the "sword", that is an elongated colored extension of the ventral rays of the caudal fin. Some species have very long extended colorful swords that can be even longer than the body of the males (Rosen 1960; Kallman & Kazianis 2006). The sword is an evolutionary novelty in this genus and its origin and evolutionary history have extensively been investigated in a phylogenetic context (Meyer *et al*. 1994; Meyer 1997; Jones *et al*. 2013; Kang *et al*. 2013). The origin of sword has been under debate for several decades. One of the hypotheses explaining the origin of the sword is the pre-existing bias hypothesis that female preference (or sensory bias) for sword already existed before the appearance of sword, which drives the evolution of the sword in several swordtail species (Basolo 1990a, 1995a). This hypothesis was supported by the fact that several platy fish females, of which males do not carry sword, still show a preference for males with artificial sword (Basolo 1990a). Another closely related and ancestral species, poeciliid fish *Priapella olmecae* females showed the preference for sword, further supporting the pre-existing bias hypothesis (Basolo 1995b). More recently, comprehensive phylogenetic analyses of *Xiphophorus* suggested that the sword existed in a common ancestor in this genus and was lost secondarily in platies multiple times independently (Meyer 1997; Kang *et al*. 2013; Jones *et al*. 2013).

Genes or genetic pathways involved in the development of sword have been identified in several studies. Candidate gene approach revealed that several genes such as *msx* and *fgfr1* are expressed in the developing sword under hormone treatment in a swordtail species, *Xiphophorus hellerii* (Zauner *et al*. 2003; Offen *et al*. 2008) (Table 4.1). Recently, Kang *et al* (2015) investigated gene expression changes in the developing sword at the whole

transcriptome levels using high-throughput RNA-Seq in the swordtail, *X. hellerii*. That study provided a catalogue of candidate genes to understand the architecture of gene regulatory networks of the development of the sword. A large number of differentially expressed genes (1,784) in hormone-induced sword highlight the massive changes that are taking place during the development of sword (Kang *et al*. 2015). Interestingly, many embryonic developmental genes were involved in the sword development and approximately 70 % of those differentially expressed genes were shared by another male specific and evolutionary older trait, gonopodium. These findings suggest that genetic networks are "co-opted" during the development and evolution of gonopodium, and are subsequently deployed as well in the later evolution of another novelty, the sword (Kang *et al*. 2015).

2.4. Colorful sexual traits

Coloration on the sexual ornaments are often regarded as the evolutionary outcomes of sexual selection since it represents a honesty signal of individual quality of mate (e.g. male condition and genetic quality) and can thus be used by females for choosing their partner. Examples include plumage and melanin colorations in birds (Keyser & Hill 2000; Badyaev *et al*. 2001; Laucht *et al*. 2010) and pigmentation patterns in African cichlid fishes (Seehausen *et al*. 1999) and guppies (Kottler *et al*. 2014). In particular, carotenoid pigment that is responsible for yellow, orange and red colorations has been suggested as an indicator of various health conditions reflecting male quality in many fish and birds (McGraw 2005). For example, it indicates males' nutritional conditions (Grether 2000) or parasite resistance (Kolluru *et al*. 2006) in guppies. Another carotenoid-based ornament, red nuptial color, in male sticklebacks was suggested to be a honest signal of male quality regarding antioxidant utilization (Pike *et al*. 2007). Extraordinarily sexually dimorphic male coloration in the ring-necked pheasant also reflects nutritional health conditions in their early life (Ohlsson *et al*. 2002). Moreover, carotenoid based coloration of bill in house sparrow (Laucht *et al*. 2010) and plumage in house finches (Badyaev *et al*. 2001) reflect the male quality. A positive genetic correlation between beak color and immune function was found in zebra finches (Birkhead *et al*. 2006). Other pigment-based colorations such as melanin- or pterin-based coloration of ornaments also play a role in the evolution of sexually selected traits. Level of antioxidants in egg yolk showed a positive relationship with female ornaments, or pterin-based orange color patches in lizards (Weiss *et al*. 2011). Phylogenetic studies of Australian dragon lizards revealed the essential role of sexual selection in the evolution of color pattern complexity (Chen *et al*. 2012).

The genetic mechanisms of the different pigmentation-based body colorations have been investigated. Recent transcriptome approaches identified several potential genes such as *coatomer protein complex, subunit zeta-1* (*copz-1*) that might be involved in coloration (Gunter *et al*. 2011) and melanophore maintenance in cichlid fish (Henning *et al*. 2013). It has been shown that black ornaments of guppy males develop under the effect of *colony-stimulation factor 1 receptor* a (*csf1ra*), which mediates the xanthopore-melanophore interaction (Kottler *et al*. 2013). Transcriptomic studies of the carotenoid pigmentation provided a candidate gene list including expression of *Eorix* proteins in a carotenoid-signaling bird species (Pointer *et al*. 2012). Genes responsible for beak color in zebra finches were identified using QTL (Quantitative Trait Loci) analyses (Schielzeth *et al*. 2012) and also genes for melanin-containing organelles (melanosomes) were found (Maia *et al*. 2013). Hox gene pathways were found to be involved in sexually selected pigmentations in *Drosophila* species (Kopp *et al*. 2000; Jeong *et al*. 2006). In wild guppy populations, multilocus heterozygosity (MLH) was suggested to be as a significant predictor of the orange spot in males that is sexually selected trait by female preference (Herdegen *et al.* 2013).

2.5. Sexually selected behavioral traits (e.g., call, song)

Social behavior (or communication signal) can be a target of sexual selection, because its certain type might be preferred by the opposite sex for mating. In birds and amphibians, male's mating calls and female's responsive calls to them are often important acoustic social signal for their mating. Various modes of social behaviors such as species recognition, mating preference, foraging behavior, courtships, and male dominance hierarchy have been investigated to identify genes or genetic mechanisms controlling those traits (reviewed in Robinson *et al*. 2008). For example, genes such as *egr1* for species recognition in songbirds and song recognition in zebra finches, and *per* for the courtship communication in fruit flies were identified (Robinson *et al*. 2008). The links of several genes to mating behavior were also determined. Expression of *lov-1*, a polycystic kidney-disease gene (PKD1) homologue that is known to control mating behavior, was detected in sensory neurons in males of the nematode, *Caenorhabditis elegans* (Barr & Sternberg 1999). Recently, genetic architecture of male song, a sexually selected trait, in an aquatic moth, *Achroia grisella*, has been studied using a QTL approach (Limousin *et al*. 2012). Genes regulating sexually selected behavior (or mating calls) might be different from ones controlling morphological traits. Alternatively, mating preference and sexually selected morphological traits have a common genetic basis through genetic linkage (Smith 1966). Genes or genetic pathways related to female mate

choice have been identified (Chenoweth & McGuigan 2010). A recent review suggested that sexually selected traits are more susceptible to environmentally-induced epigenetic alterations than naturally selected traits (Jasarevic *et al*. 2012).

3. Common genetic architecture among sexually selected traits

Recent investigations on diverse sexually selected traits or exaggerated male ornaments provided interesting insight into their genetic backgrounds or genetic mechanisms. Intriguingly, accumulating information about genes or genetic pathways accounting for sexually selected traits/exaggerated ornaments revealed several groups of genes that are commonly involved across distantly related animal lineages. For example, conserved genes are expressed in eye-antennal disc in both *Drosohophila* species and stalk-eyed flies (Warren & Smith 2007). Water strider (*Aquarius remigis*) and *Drosophila* species diverged around 371.9 mya ago (www.timetree.org) (Hedges *et al*. 2006), but they still exploit the same genetic mechanisms to develop sexually selected traits. Although the shared genes might not be regulated in the same way across traits and taxa, the findings of the common genetic architecture underlying diverse sexually selected traits in different animal lineages provide valuable insight for a better understanding of their developmental origin and evolutionary history.

3.1. Signs of co-option

The recruitment of pre-existing genetic network systems for new purpose using "gene network co-option" has been suggested to be a usual way for the development and evolution of morphological novel traits (Carroll *et al*. 2008; Fraser *et al*. 2009; Monteiro & Podlaha 2009). Several recent empirical studies showed that the co-option of certain gene networks (i.e., developmental gene networks) underlies the developmental origin of novel traits. For example, the co-option of genes or regulatory network related to anteroposterior head patterning for proximodistal appendage patterning in fruit flies (Lemons *et al*. 2010), eye-developmental genes (i.e., *optix*) for red patches of pigmentation on butterfly wings (Monteiro 2012) and also for various wing scales as a novel trait contributing to speciation (Martin *et al*. 2014) has been identified. Similar patterns were also observed in the development of male exaggerated ornaments in other animal groups (Moczek & Rose 2009). Moczek *et al.* (2009) showed that limb patterning genes (distal-less and homothorax) that play an essential role in the limb development of other insect species regulate the horn

development in beetle horns, although horns are not modified at all from mouthparts or limbs. Many studies on the genetic basis of sexually selected traits found that a handful of specific genes are repeatedly involved in different sexually selected traits. The co-option of *Hox* complex has been shown to contribute to cephalopod-specific organs (Lee *et al*. 2003). *Hox* genes are well-known principal transcriptional regulators of animal body regionalization during embryonic development (Meyer & Málaga-Trillo 1999; Kopp 2011; Tanaka *et al.* 2011). *Hox* genes have also been suggested to be key-players in the development and evolution of novel complex traits such as male genitalia (i.e. imaginal discs) (Estrada *et al.* 2003), a secondary sexual trait - sex combs in *Drosophila* species (Barmina & Kopp 2007) and beetle horns (Wasik *et al.* 2010). Transcriptomic profiling of the sword in *Xiphophorus hellerii* showed that many *hox* genes (i.e., *dlx*, *lhx9*, *satb2*, *zhx2* and etc.) are involved in its development (Kang *et al*. 2015). Another *hox* gene, distal-less (*dlx* or *dll*), which is known to be related to the morphological development, was shown to regulate the development of beetle horns (Moczek & Rose 2009), antenna in water strider (Khila *et al*. 2012), mandible in stag beetle (*cmdsx*) (Gotoh *et al*. 2014), eyespot size in butterfly (Monteiro *et al.* 2013) and sword in swordtails (Kang *et al*. 2015). This gene is differently expressed according to species, sex, body regions and size in beetle horn (Moczek & Rose 2009). *Dlx* paralogs have also been found to be candidate genes for evolutionary innovations in cichlid fish (Diepeveen *et al*. 2013). An analysis of male sex comb regions showed that *dlx* expression is significantly correlated with other *hox* genes (Tanaka *et al*. 2011). *Scr* also belongs to another *hox* gene family and its role is known to regulate the segment identity in many insect groups including *Drosophila melanogaster* (Struhl 1982; Curtis *et al*. 2001). *Scr* is also found to be expressed in the development of various sexually selected traits (Zauner *et al.* 2003; Barmina & Kopp 2007; Williams *et al.* 2008; Masse *et al*. 2009; Wasik *et al*. 2010).

Bone morphogenetic proteins (*bmp*) are known to play an essential role in many different, but important developmental pathways (Hogan 1996). Expression of *bmp-3b* (Kapanen *et al*. 2002) and *bmp2* (Feng *et al*. 1997; Pita-Thomas *et al*. 2010) is detected in the deer antler, which is a male weapon used for male-male competition for mating. The pleiotropic effects of *bmp2* and *hao1* have been identified in the comb mass, a sexual ornament in chickens by QTL mapping (Johnsson *et al.* 2012). Recently, comprehensive transcritpomic analysis using RNA-Seq has found the expression of *bmp1* in the developing sword in swordtail fish (Kang *et al.* 2015). Moreover, several *bmp* are involved in the development of evolutionarily and ecologically important traits such as the beak of Darwin's finch, which is a classical example of adaptive radiation (i.e., correlation of expression of *bmp-4* for the beak morphology)

(Campas *et al*. 2010). A comparative analysis of expression patterns of growth factors showed that bone morphogen has a strong correlation with the expression of *bmp-4* in various beak shapes in Darwin's finch (the genus *Geospiza*) (Abzhanov *et al*. 2004). These empirical data support the co-option of *bmps* in the expression or development of the sexually selected or exaggerated traits. Those genes found in the development of diverse sexually selected traits or exaggerated male ornaments are indeed key regulators for the embryonic development and also for the development of non-sexually selected (normal) body parts. Therefore, we hypothesize that the co-option plays an important role in the development and evolution of sexually selected traits in general.

3.2. Sex determination and sex-biased genes

It has been suggested that the coordination of sex-specific development assists in the evolution of sexual traits and the gene regulatory network governing the sexual development (Chenoweth & McGuigan 2010). Sex-determination systems are enormously diverse, but their downstream components of are generally known more evolutionarily conserved (Williams & Carroll 2009). Sex determination systems induce and coordinate sexual differentiation of gonads during developmental processes, leading to sex-specific differences (sexual dimorphism) in various phenotypic features such as behavior, morphology and physiology. In general, sexual differentiation can be regulated by sex chromosomes in a cell-autonomous fashion or by a sex-specific hormone signal received from the gonads or other related tissues (Bear & Monteiro 2013). A few reported genes related to a function of sex determination are known, including *sex-determining region on Y* (*sry*) in mammals, *dmrt1* in birds, *dmy, gsdf* and *amhr2* in fish (reviewed in Trukhina *et al*. 2013). A majority of sex-determination genes are known to be typically involved in the development of primary sexual traits (i.e. gonad). However, more and more evidences are accumulating that those genes are also activated in the development of "secondary" sexual traits (i.e. sexually selected traits). For example, *doublesex* (*dsx*), which is a well-known key regulator for the sex determining cascade gene in insects, is found to regulate the development of sexually dimorphic traits in fruit flies, mammal, birds and fish (Williams & Carroll 2009). *Dsx* functioned as a regulator controlling various aspects such as dimorphisms between sexes, morphs and species in the development of beetle horns (Kijimoto *et al*. 2012). This gene is also involved in the development (Struhl 1982; Dworkin 2005) and the sex specific expression of sex comb in *Drosophila* species. Expression patterns of *cyclommatus metallifer dsx* (*cmdsx*) in the sex-

specific mandible growth in male stag beetle were shown to be mediated by Juvenile hormone (JH) signaling pathways (Gotoh *et al*. 2014).

Several *sex determining region on Y* (*sry*) are generally known to be involved in the development of primary sexual characters (i.e. testis development). Nevertheless, those genes are also found to be expressed during the development of secondary sexual characters. Several *sox* genes (*sox* 2, 3, 5, 9 and 10) were shown to be involved in the sword development (Kang *et al*. 2015). Transcriptome analysis of Chinese sika deer antler identified the expression of *SRY-box 9* (*Sox9*) during rapid growth in the antler development (Yao *et al*. 2012b). Especially, *Sox9* (*sry-box 9*) is a well-known sex-determination gene (Sekido and Lovell-Badge 2008). Its conserved role during gonad development in vertebrates (Yokoi *et al*. 2002) and its association with testis differentiation in mouse (Wainwright *et al*. 2013) and other mammals (Barrionuevo *et al*. 2012) are well documented. Other genes such as *Inhbb* (Yao *et al*. 2006), *cyps*, and g*ps7*, *8* (Zheng *et al*. 2013), which previously demonstrated sex-specific divergent expression patterns in other animal groups, were also differentially expressed in the developing sword in swordtail fish (Kang *et al.* 2015).

It has been suggested that rapidly evolving male-specific traits in taxa (e.g. *Xiphophorus* and *Poecilia* in fish) are less likely to be affected by sex-determining systems given no significant relationship between the evolution of male ornaments and sex chromosome was found in comparative phylogenetic analyses (Mank *et al*. 2006). However, a growing body of expression data on sex-determination and/or sex-biased genes highlights their potential role in the development and evolution of secondary sexual traits.

3.3. Insulin signaling pathway and growth related genes

Higher sensitivity of cells to insulin/insulin-like growth factor (IGF) signaling pathways in a male exaggerate trait (e.g., horn) than other body parts (e.g., genitalia and wings) in rhinoceros beetle horns suggests that IGF signaling genetically controls the horn development and generates an honest signal of male nutritional conditions (Emlen *et al*. 2012). It has been suggested that the extreme growth of horn in rhinoceros beetle is by-product of the growth mechanisms and demonstrates an important role of the IGF pathways in the horn development. Several growth factor related genes are found to be expressed in the development of diverse sexually selected traits. Growth factor related genes such *transforming growth factor, beta 3* (*tfgb3*), *insulin-like growth factor 2* (*igf2*), *insulin-like growth factor binding protein 4* (*igfbp4*) and *IGF-like family receptor 1* (*igflr1*) were differently expressed in the developing sword in swordtail fish (*Xiphophorus hellerii*),

compared to other tissues (Kang *et al*. 2015). GO terms such as growth factor activity and response to growth factor stimulus were also enriched in the developing sword. Several studies of deer antler indicated that *igf-1* acts as an antler growth stimulus (AGS) (Suttie *et al*. 1985, Sadighi *et al*. 1994; Sadighi *et al*. 2001) and *igf-1 receptor* is expressed in the tip of growing antler (Elliott *et al*. 1993). Growth factors such as *igf*-1, *ngf* and *nt3* are also observed to be expressed in the deer antler during regeneration (Pita-Thomas *et al*. 2010). A comprehensive transcriptome study in Sika deer antler found that *igf* II is significantly highly expressed (Zhao *et al*. 2013). Male-specific body color patterns including tails characterize sexual dimorphism in guppies (Endler 1984). A transcriptome analysis of the guppy (*Poecilia reticulata*) showed that male-biased expressed genes in tails are enriched with GO term related to insulin receptor binding (Sharma *et al*. 2014). Expression of genes involving the insulin and IGFs signaling pathways in various male exaggerated ornaments across several animal groups might indicate that different sexual traits also use the same genetic mechanisms for their development processes as suggested in beetle horn. Furthermore, the action of those pathways in fact modulates the growth of organisms in response to the changes of nutritional and physiological conditions. Exaggerated traits as an honesty signal for male quality such as nutritional conditions and physiological states would support this idea.

Other growth-related hormonal regulation observed in sexually selected traits is Juvenile hormone (JH). JH is a key hormone to regulate the embryonic development and the postembryonic metamorphosis in insects (Truman & Riddiford 2007). The growth of exaggerated male traits has been well examined in insects (Lavine *et al*. 2015). Size effects of perturbations to juvenile hormone on exaggerated traits such as stalk-eyed flies (Fry 2006), mandibles of stag beetle (Gotoh *et al*. 2011), flour beetle (Okada *et al*. 2012), and horns of dung beetles (Emlen & Nijhout 2000; Moczek & Nijhout 2002; Shelby *et al.* 2007) have been reported. Weapon-specific sensitivity to JH signaling has been identified in the dung beetle and stage beetle (Emlen & Nijhout 1999; Gotoh *et al*. 2011). Furthermore, it was suggested that differential responsiveness to JH influences the intensity of sexual dimorphism e.g., weapon sizes since JH titer during the JH sensitive period was not different between sexes (Gotoh *et al*. 2011). JH-mediated allocation of resources to eye-span in stalk-eyed flies at the expense of the development of testes indicates the potential fitness costs for the production of an exaggerated trait (Fry 2006).

3.4. Steroid hormone related genes

The role of steroid hormones (e.g., androgens, estrogens and glucocorticoids) in the expression or development of sexually selected traits has been extensively examined in several taxa. A positive relationship was found between levels of steroid hormones and sizes of sexual ornaments. For instance, badge size in house sparrows is associated with levels of testosterone (Laucht *et al*. 2011) and the size of a melanin-based black bib in male house sparrow with glucocorticoid receptor (Lattin & Romero 2013). A regulation of testosterone on the development of deer antler was also identified (Suttie *et al*. 1995). Whether levels of testosterone and corticosterone predict the dewlap size in males of the green anole lizards (*Anolis carolinensis*), which is a predictor of bite-force capacity, was also tested (Husak *et al*. 2007). The relationships between the hormone levels and the ornament size in this lizard species vary according to their body size (e.g. heavy weight and light weight) (Husak *et al*. 2007).

It has been shown that sex hormone related genes are involved in the development of various sexual ornaments directly or indirectly. Androgen modulated expression has frequently been identified in several sexually selected colorations such as red gular pouch coloration (skin ornament) in a frigate bird (Madsen *et al*. 2007), sexually dimorphic facial hair coloration in red-fronted lemurs (Clough *et al*. 2009), enlarged red eye ring (more pronounced in males) in diamond doves (Casagrande *et al*. 2011) and male plumage color in red-backed fairy-wrens (Lindsay *et al*. 2011). However, direct examples showing the expression of genes involved in androgen pathways in sexually selected traits or male ornaments are rather sparse. Expression of *androgen receptor β* (*arβ*) was found in the developing swords in *Xiphophorus hellerii,* which is a swordtail species possessing a long colorful sword (Zauner *et al*. 2003; Offen *et al*. 2008; Kang *et al*. 2015).

Less attention has been paid to the effects of glucocorticoids on the expression and development of elaborated male traits relative to the aforementioned effects of androgens. Recently, however, glucocorticoids mediated mechanisms on the expression have been reported in several male-specific traits and both steroids of glucocorticoid and androgen were suggested to be simultaneously responsible for the expression of the sexual traits (Leary & Knapp 2014). Differential expression of *glucocorticoid induced transcript 1* (*GLCCI1*) in the developing sword in *X. hellerii* would further support its significant role in the development of male ornaments.

Estrogen levels have an influence on the regulation of sexual ornaments in males as well as female-specific ornaments. Sex-specific regulation of estrogen pathways rather than

androgen or insulin growth factor signaling pathways is suggested to be a primary regulator factor in affecting male-biased polymorphism, long faces in males in Anolis lizard (Sanger *et al.* 2014). Similarly, several estrogen-related genes are found to be up-regulated in male specific traits of the sword in swordtail fish (Kang *et al*. 2015). Previously recognized genetic mechanisms underlying sexual dimorphism need to be further examined (Casagrande *et al*. 2011). Steroid hormone signaling pathways would be one of the intriguing future avenues on the genetic mechanisms underlying male specific sexual traits.

3.5. Other reported common genes

Beside the genes or genetic pathways mentioned above, several other genes are commonly expressed in sexually selected traits among distantly related lineages. For example, *crooked legs* and *cdc2* are expressed in the eye-antennal imaginal discs in stalk-eyed flies (Baker *et al.* 2009). Zinc finger proteins, homologs of *crooked legs* and several *CDCs* (e.g., *CDC7*, *16*, *20*, *27* and *34*) are also up-regulated in the developing sword in *X. hellerii* (Kang *et al*. 2015). *Fork head* (*fkh*) genes (e.g., *FOXs*) that are natural target of *sex combs reduced* (*Scr*) are expressed in sex combs in *Drosophila* species (Ryoo & Mann 1999) and also up-regulated in the sword in the swordtail (Kang *et al.* 2015).

4. Transcriptome profiling of sexually selected traits or male ornaments

Emerging deep sequencing techniques offer an opportunity to explore the genetic mechanisms or regulatory architecture underlying evolutionarily or ecologically interesting traits such as adaptive phenotypes including sexually dimorphic characters or sex-specific (often male-specific) ornaments in non-model organisms. Transcriptome studies on the sex *per se* have increasingly been published in recent years and they provide a fundamental resource for the sex-specific or sex-biased gene expression patterns in various animal taxa (Elmer *et al*. 2010; Assis *et al*. 2012; Naurin *et al*. 2012; Wilkinson *et al*. 2013; Albritton *et al*. 2014; Sharma *et al*. 2014). This information of sex-specific gene expression data can also be used as a valuable resource to explore the genetic regulatory background underpinning the development of sexually selected traits or male-specific ornaments. Although only few transcriptome studies that looked directly into the sexually selected traits are currently available (relative to transcriptome studies on the sex *per se*), it is worthwhile to review them to get an overview of transcriptome profiles of the sexually selected traits (Table 4.2). One general pattern is that most of transcriptomes expressed in sexually selected traits showed a great number of genes undergone changes in the expression during their development. For

example, for a transcriptome analysis on the development of sika deer antler 5,573 genes (out of 16,905 significantly changed transcripts) are differentially expressed at two different developmental stages (Zhao *et al*. 2013). Developing sword in swordtail fish showed that 1,782 genes are differentially expressed compared to the control fin rays under hormone treatment condition and several sword specific genes were also identified (Kang *et al*. 2015). Transcriptome profiles can also provide information on trait-specific gene expression patterns or particular genetic pathways that are mostly represented in traits of interest. For instance, transcriptome analysis of sika deer antler revealed that genes and genetic pathways related to protein synthesis and translation (i.e. elongation factors) are most significantly changed during development (Zhao *et al*. 2013). Sword transcriptome showed that embryonic organ development, sexual character development and coloration genes were significantly highly expressed (Kang *et al*. 2015). Kang *et al*. (2015) further suggested co-opted genetic networks for the development of sword and another male specific sexual trait, gonopodium. Furthermore, transcriptome analysis has been attempted to understand the pattern or mode of evolution in sexually selected traits. For example, two mechanisms such as positive selection and developmental decoupling, which are critical for the rapid evolution of extreme sexually selected traits, have been investigated by comparative transcriptome analyses of the two scarab beetles (Warren *et al*. 2014). These two scarab beetles show differences in the horn location (head vs. thorax), shape and the response of horn growth to nutrition. Genes with horn-biased expression and those with morph-biased expression (e.g., horned males vs. horn-less males) are suggested to have undergone positive selection and relaxed purifying selection, respectively (Warren *et al*. 2014). Positive selection on sexually selected traits is found in only a few genes while relaxed purifying selection resulted in more broadly distributed genetic variation in several genes (sex- or morph- specific expression of ornaments and weapons) (Warren *et al*. 2014).

We can also identify some of genes or genetic pathways shared between transcriptome profiles of two different sexually selected traits in distantly related lineages. In the transcriptomes of two sexually selected traits, the sword in swordtail (Kang *et al*. 2015) and the tail in guppies (Sharma *et al*. 2014), several GO terms such as plasma membrane (GO:0005886), cellular biogenic amine metabolic process (GO:0006576), carbohydrate transport (GO:0008643), neuropeptide signaling pathway (GO:0007218) and melanosome (GO:0042470) are commonly found. Interestingly, we also found that many genes or groups of genes aforementioned on other sexually selected traits such as color gene [*xanthine dehydrogenase* (*xdh*) and *premelanosome protein (pmel*)], hox genes [*ALX homeobox 4*

(*alx4*)], hormone gene [*adrenoceptor alpha 2B*], growth factor related genes [*growth hormone receptor* (*ghr*), *insulin receptor substrate 2* (*irs2*), *insulin-like growth factor 2 mRNA binding protein 3* (*ifg2bp3*), sex determining genes (*sox8*, *9*, *10*) and *pax9* are all commonly found in both sexually selected traits. Transcriptome investigations on the sexually selected traits have still been limited. However, emerging transcriptome analyses would provide important resources for future research on investigating conserved genes or genetic pathways underlying sexual traits in distantly related species at the whole transcriptional level. The information would be very useful to select candidate genes or genetic pathways to investigate the genetic mechanisms in more details to understand how sexually selected traits or male ornaments have arisen. Furthermore, accumulating comprehensive transcriptome data on these sexual traits would shed valuable insight for future research to understand the evolutionary origin and the genetic mechanisms of the development of evolutionary novelties more widely.

5. Concluding remarks

We reviewed the genes, genetic mechanisms and regulatory pathways found in recent studies that investigated the genetic basis of sexually selected traits or exaggerated male traits in diverse animal groups. Currently, more and more genetic information about sexually selected traits or male-specific traits is emerging and accumulating with advanced sequencing technologies. The amount of information is not sufficient so far to comprehend a whole story of the development and evolution of these traits. However, common genes and genetic architectures shared among different sexually selected traits across distantly related animal lineages will provide a framework for future research on uncovering their origin and evolution. Further, considering those commonly expressed genes would be critical for future studies to identify upstream or down stream regulators of sexually selected or male exaggerated traits and also to determine the transcriptional changes of these genes in these traits.

Table 4.1 Genes identified to be involved in the sexually selected traits or male ornaments from studies using candidate gene approaches.

Trait	Organism	Genes	Features	References
Eye span (eye-antennal disc)	Salk-eyed flies *(Cyrtodiopsis dalmanni* and *Sphyracephala beccarn)*	*Distaless (dll), hedgehog* (*hh*), *wingless* (*wg*), *engrailed* (*en*), *and defective proventriculus* (*dve*)	-	(Hurley *et al*. 2001; Warren and Smith 2007)
	Stalk-eyed flies (*Teleopsis dalmanni*)	*Crooked legs* and *cdc2*	EST (Expressed Sequence Tag) sequencing and microarray analysis	(Baker *et al*. 2009)
Sex comb	Fruit fly (*D. melanogaster*)	*Sex comb reduced* (*Scr*), *doublesex* (*dsx*)	Sex comb morphogenesis	(Tanaka *et al*. 2011; Devi & Shyamala 2013)
		Daschund (*dac*) and *distaless* (*dll*)	Sex comb development	(Atallah *et al*. 2014)
	Fruit fly (*D. mauritiana* and *D. simulans*)	*Dsx*	Sex comb divergence	(Graze *et al*. 2007)
	Fruit fly (*Drosphila* species)	*Scr*	-	(Barmina & Kopp 2007; Graze *et al*. 2007; Williams *et al*. 2008; Wasik *et al*. 2010)
Male-specific abdominal pigmentation	Fruit fly (*D. melanogaster*)	*Bric-à-brac* (*Bab*) and *dsx*	Genes from pre-existing dimorphic traits	(Williams *et al*. 2008)
Horn (weapon)	Rhinoceros beetle	*Hh*, *wg* and *dpp*	Determination of precise location of the horn outgrowth	(Reviewed in Emlen *et al*. 2006)
	Onthophagus taurus and *O. binodis*	*Dlx* or *dll*	Tissue specific expression	(Moczek & Rose 2009)
	Rhinoceros beetles (*Trypoxylus dichotomus*)	*Insulin/insulin-like growth factor*	Tissue specific expression	(Emlen *et al*. 2012)

	Rhinoceros beetle (*Onthophagus taurus* and *O. sagittarius*)	*Dsx*	Morph-, sex- and species-specific development	(Kijimoto *et al*. 2012)
Comb mass (sexual ornament)	Chicken (White Leghorn chickens and a Red Junglefowl)	*Bmp2* and *hao1*	Pleiotropic effects	(Johnsson *et al*. 2012)
Sword	Swordtails fish (*X. hellerii*)	*Msx* and *fgfr1*	Hormone induced	(Zauner *et al*. 2003; Offen *et al*. 2008)
Male black ornament	Guppy (*Poecilia reticulata*)	*Colony-stimulation factor 1 receptor* a (*csf1ra*) and *Kita*	Pigment pattern formation	(Kottler *et al*. 2013).
Antler	Deer species	*BMP-3b* and *BMP2*	-	(Feng *et al*. 1997; Kapanen *et al*. 2002; Pita-Thomas *et al*. 2010)

Table 4.2 Recent genome-wide transcriptomic studies on the sexually selected traits.

Trait	Organism	Features	Method	References
Horn	*Onthophagus* beetles	Development of horn	EST and microarray	(Kijimoto *et al*. 2009)
	Horned beetle (*Onthophagus Taurus*)	Whole body including horn	EST (454 pyrosequencing)	(Choi *et al*. 2010)
		Development of horn (head horns and thoracic horns)	Microarray	(Snell-Rood *et al*. 2011)
	Asian rhinoceros beetle (*Trypoxylus dichotomus*) and dung beetle (*Onthophagus nigriventris*)	Phenotypically plastic traits (Horn biased gene expression)	RNA-Seq	(Warren *et al*. 2014)
Antler	Sika deer (*Cervus Nippon hortulorum*)	Endochondral ossification (Ossification stages)	RNA-Seq	(Yao *et al*. 2012a)
		Regeneration and rapid growth (Antler's tip)	RNA-Seq	(Yao *et al*. 2012b)
		Differential developmental stages (60 and 90 days)	RNA-Seq	(Zhao *et al*. 2013)
Plumage coloration	Ruff (*Philomachus pugnax*)	Plumage coloration and mating strategies	RNA-Seq	(Ekblom *et al*. 2012)
Sword	Swordtail fish (*Xiphophorus hellerii*)	Development of male ornament and sexual organ under the hormone treated condition	RNA-Seq	(Kang *et al*. 2015)

Summary

The objectives of my Ph.D. dissertation were to understand the evolutionary history of the genus *Xiphophorus* fish species from Mesoamerica and to determine the molecular genetic basis of the development of the sexually selected traits, sword and gonopodium (a male intromittent organ), in *Xiphophorus hellerii*.

In **Chapter 1**, I conducted a comprehensive phylogenetic analysis of all 26 known *Xiphophorus* species including four recently described species (*X. kallmani*, *X. mayae*, *X. mixei* and *X. monticolus*) using two mitochondrial and six new nuclear markers. The mitochondrial and nuclear maker-based phylogenetic trees revealed discordance in the position of one of the newly described species, *X. monticolus,* suggesting that this species is likely to have arisen through an ancient hybridization event. This finding highlights the potential key role of hybridization in the evolution of this genus and suggests the need for further investigation of how hybridization contributes to speciation more generally. The evolutionary history and character state evolution of the sword were reconstructed in this study based on the phylogeny. The ancestral state reconstruction of the sword, particularly based on the maximum likelihood approach, strongly suggests that the common ancestor of the genus *Xiphophorus* already possessed a sword and that it was lost completely secondarily again in the derived platy lineage of the genus. My complete molecular phylogeny of the genus *Xiphophorus* provides a comprehensive phylogenetic framework to understand various evolutionary processes in this genus.

In the next two chapters (Chapters 2 and 3), I focused on the developmental molecular basis underlying two evolutionarily important traits, the sword and the gonopodium in a swordtail fish, *Xiphophorus hellerii*. In **Chapter 2**, I investigated whether androgen signaling and retinoic acid (RA) signaling pathways are involved in the development of gonopodium. We found that retinaldehyde dehydrogenase (*aldh1a2*), a RA synthesizing enzyme, and two retinoic acid receptors, *rar-ga* and *rar-gb* are expressed in the developing gonopodia by *in situ* hybridization. To better understand the dynamics of RA signaling during the metamorphosis of the anal fin towards a gonopodium I further examined the regulation of *aldh1a2* expression by real-time quantitative PCR (qPCR). Induction of *aldh1a2* expression

was proportional to the rate of metamorphosis of anal fin to gonopodium. To test whether RA signaling is a potential major determinant of gonopodial development more directly, I enhanced RA signaling and examined its effect on the gonopodium. Furthermore, We found that *androgen receptor* (*ar*) expression overlaps with *aldh1a2* and *rarg* expression in the growing tips of anal rays that are eventually incorporated into the mature gonopodium. These findings suggest that the sex steroid-controlled metamorphosis of the anal fin towards a gonopodium is mediated by an increase in RA signaling. This study highlights the role of RA signaling, which is a well-known morphogen during embryonic development, in post-embryonic metamorphosis.

In **Chapter 3**, I performed transcriptome analyses of the sword and gonopodium in *Xiphophorus hellerii* using the RNA-Seq technique to determine the genetic networks involved in the development of these male-specific traits. I identified a large number of genes with tissue-specific expression patterns in hormone-induced sword and gonopodium: 5,433 and 1,784 of differentially expressed genes for the gonopodium and the sword, respectively highlight the massive changes that are taking place during the transformation of the anal fin to a gonopodium and of the ventral caudal fin to a sword. I also identified sword- and gonopodium-specific gene expression patterns. A large number of genes were shared between the development of sword and gonopodium, and many embryonic developmental genes were differentially expressed. These results indicate that genetic regulatory networks are co-opted during the development and evolution of gonopodium, and were subsequently deployed as well in the later evolution of another novelty, the sword. This study provides a catalogue of candidate genes and will serve as basis for future efforts to dissect the genetic mechanisms underlying the development of male-specific traits in swordtails, in other male-specific traits, and even in sexually selected traits in animals generally.

From Chapter 3, I found that several genes involved in the development of the sword are shown to be the same genes or gene families that are known to be involved in other male exaggerated or sexually selected traits in distantly related taxa. Therefore, it is worth to compare genes and genetic pathways among different sexually selected traits to test whether common (or shared) genetic mechanisms contribute to the evolution of those traits. In **Chapter 4,** I review the emerging empirical data on the genetic mechanisms underlying sexually selected traits to examine whether common or shared genetic mechanisms underlie those traits across different taxa. In this chapter, I summarizied the genes and regulatory

pathways underlying various types of sexually selected traits, exaggerated male ornaments, and complex sexual traits among distantly related animal groups. Then, I discussed various intriguing aspects of the common genetic architecture or the gene expression underlying those traits, such as signs of co-option and the role of sex-determination/sex-differentiation system. An investigation of the common genetic architecture underlying sexually selected traits provides valuable insight for a better understanding of the origin and evolution of sexually selected traits.

The major scientific contributions of this dissertation include 1) a comprehensive phylogenetic investigation to elucidate the phylogenetic relationships among the entire species in the genus *Xiphophorus* and the evolutionary history of the sword, 2) identification of the role of retinoic acid signaling in the developing gonopodia, 3) a genome-wide expression profile in the sexually selected male-specific traits, sword and gonopodium in *Xiphophorus hellerii* and 4) a summary of emerging empirical data on the genetic mechanisms underlying sexually selected traits to look at the common genetic mechanisms underlying those traits.

Zusammenfassung

Die Hauptziele meiner Dissertation bestanden darin, die evolutionäre Geschichte der mittelamerikanischen Fischgattung *Xiphophorus* nachzuzeichnen und die Entwicklung von Schwert und Gonopodium, zweier sexuell selektierte Merkmale, in der Art *Xiphophorus hellerii* auf molekularer Basis zu untersuchen.

Im ersten Kapitel habe ich eine umfangreiche phylogenetische Analyse aller 26 beschriebenen Xiphophorus-Arten durchgeführt, inklusive der vier kürzlich beschriebenen Arten *X. kallmani*, *X. mayae*, *X. mixei* and *X. monticolus.* Für diese Analyse wurden zwei mitochondriale und sechs nukleare Marker verwendet, wobei das Ergebnis zwei kontroverse phylogenetische Stammbäume (mitochondriale vs. nukleare Marker) in der Position der Art *X. monticolus* hervorbrachte. Dieses Ergebnis weist darauf hin, dass die Art durch Hybridisierung entstanden sein könnte und unterstützt die Theorie einer möglichen Schlüsselrolle von Hybridisierung in der evolutionären Geschichte der Gattung *Xiphophorus*. Diese molekulare Phylogenie bietet einen umfangreichen phylogenetischen Rahmen zum Verständnis diverser evolutionärer Prozesse in der Gattung *Xiphophorus*.
Weiters wurde aufbauend auf die Phylogenie die evolutionäre Geschichte zur Entstehung des Schwertes rekonstruiert. Basierend auf einer Maximum-Likelyhood-Methode ergab die Rekonstruktion des ancestralen Merkmals, dass der gemeinsame Vorfahr der Gattung *Xiphophorus* bereits ein Schwert besaß welches sekundär in der Linie der Platies wieder verloren ging.

In den Kapiteln 2 und 3 beschäftigte ich mich mit den zwei evolutionsbiologisch sehr wichtigen Merkmalen, nämlich dem Schwert und dem Gonopodium im Schwertträger *Xiphophorus hellerii.* Hierfür habe ich die entwicklungsbiologische Entstehung dieser Merkmale auf molekularer Ebene näher analysiert.

Im zweiten Kapitel habe ich untersucht, inwiefern Androgenrezeptor-Signalwege (AR) und Signalwege der Retinolsäure (RA) in der Entwicklung des Gonopodiums involviert sind. Ich habe herausgefunden, dass Retinaldehyd-Dehydrogenase (*aldh1a2*), ein RA-synthetisierendes Enzym, und die Retinolsäurerezeptoren *rar-ga* und *rar-gb* in den sich

entwickelnden Gonopodien exprimiert werden. Um die Dynamik der RA-Signalwirkung während der Metamorphose von der Analflosse zum Gonopodium besser zu verstehen, habe ich die Regulation der *aldh1a2*-Expression mit Hilfe von quantitativer Realtime PCR (qPCR) näher untersucht. Es hat sich herausgestellt, dass sich die Induktion zur Expression von *aldh1a2* proportional zum Voranschreiten der Metamorphose von Analflosse zum Gonopodium verhält. Um zu testen, ob die RA-Signalwirkung einen bestimmenden Faktor in der Entwicklung des Gonopodiums darstellt, habe ich die RA-Signalwirkung in einem weiteren Versuch experimentell erhöht.
Weiters habe ich herausgefunden, dass AR-Signale in der wachsenden Spitze der Analflosse zum Teil zeitgleich mit *aldh1a2* und *rar-g* exprimiert werden.
Diese Ergebnisse weisen darauf hin, dass die durch Steroide kontrollierte Metamorphose von der Analflosse hin zum Gonopodium durch einen Anstieg in der RA-Signalwirkung herbeigeführt wird. Generell konnte mit Hilfe dieser Studie die wichtige Rolle der RA-Signalwirkung während Post-embryonaler Metamorphose aufgezeigt werden.

Das dritte Kapitel umfasst eine Transkriptomanalyse von Schwert und Gonopodium in *Xiphophorus hellerii*. Hierfür wurde eine RNA-Seq Technik zur Untersuchung der genetischen Netzwerke herangezogen, die für die Entwicklung dieser spezifisch männlichen Merkmale mitverantwortlich sind. Ich konnte eine große Zahl an Genen mit gewebsspezifischen Expressionsmustern identifizieren: 5433 bzw. 1784 differenziell exprimierte Gene für das Gonopodium bzw. das Schwert wurden gefunden. Diese Vielzahl an Genen spiegelt die massiven Umbrüche wider, die während der Transformation von Analflosse zum Gonopodium bzw. von der ventralen Schwanzflosse zum Schwert passieren. Ich konnte jedoch auch spezifische Genexpressionsmuster für Gonopodium und Schwert identifizieren. Eine große Zahl an gleichen Genen werden beiderseits in der Entwicklung von Gonopodium und Schwert exprimiert, wohingegen viele spezifische Gene, die während der Embryonalentwicklung eine Rolle spielen, unterschiedliche Expressionsmuster in Gonopodim und Schwert aufwiesen.

Diese Ergebnisse könnten darauf hinweisen, dass genregulatorische Netzwerke während der Entwicklung und Evolution des Gonopodiums „co-opted“ wurden und später in der Evolution einer weiteren Neuheit, des Schwertes, erneut eingesetzt wurden. Diese Studie stellt einen Katalog an Kandidatengenen bereit und bietet eine Grundlage zur weiteren Untersuchung der genetischen Mechanismen, die für die Ausbildung männlicher Merkmale in den Schwertträgern verantwortlich sind.

Aus der Transkriptomanalyse konnte ich einige Kandidatengene identifizieren, die in der Entwicklung des Schwertes in *Xiphophorus hellerii* eine wichtige Rolle spielen. Frühere Studien an zum Teil weit verwandten Tiergruppen haben gezeigt, dass diese Gene in der Entwicklung männlicher bzw. sexuell selektierter Merkmale involviert sind. Daher habe ich Gene und genetische Mechanismen zwischen verschiedenen, sexuell selektierten Merkmalen verglichen um herauszufinden, ob die gleichen genetischen Mechanismen an der Evolution dieser Merkmale beteiligt sind.

Dafür habe ich im vierten Kapitel meiner Dissertation zahlreiche Studien zu einer Review zusammengefasst, um den aufstrebenden empirischen Fortschritt aufzuzeigen, der in der Erforschung eben genannter genetischer Mechanismen gemacht werden konnte. Ich habe genetische Daten über verschiedenste Typen von sexuell selektierten Merkmalen in unterschiedlichen Tiergruppen zusammengetragen und verglichen. Unterschiedliche Aspekte, von genetischer „Architektur" und Genexpression, die sexuell selektierten Merkmalen unterliegen, über Hinweise auf Co-option und die Rolle von geschlechtsdeterminierenden bzw -unterscheidenden Systemen wurden aufgeführt. Eine Untersuchung gemeinsamer genetischer „Architektur" von sexuell selektierten Merkmalen bietet wertvolle Einblicke für das Verständnis der Evolution von sexuell selektierten Merkmalen.

Die bedeutendsten wissenschaftlichen Beiträge meiner Dissertation umfassen

1.) eine umfangreiche phylogenetische Analyse, um die Verwandtschaftsbeziehungen innerhalb der Gattung *Xiphophorus* aufzuzeigen und die Evolutionsgeschichte des Schwertes nachzuzeichnen
2.) die Identifikation der Rolle von Signalwegen der Retinolsäure (RA) im sich entwickelnden Gonopodium
3.) eine genomweite Genexpressionsstudie in den sexuell selektierten, männlichen Merkmalen Gonopodium und Schwert in *Xiphophorus hellerii*
4.) eine Zusammenfassung der genetischen Mechanismen, die der Evolution sexuell selektierter Merkmale zugrunde liegen.

Record of Achievements

Chapter 1

JK conceived and designed the research, acquired all the molecular data, conducted all the statistical and phylogenetic analyses, and drafted the manuscript. MS contributed samples for the study and commented on the manuscript. RBW provided unpublished DNA sequences and commented on the manuscript. AM conceived and designed the research, participated in the coordination of the study and wrote the manuscript. All authors read and approved the final manuscript.

Chapter 2

Conceived and designed the experiments: GB NO JK AM. Performed the experiments: NO JK. Analyzed the data: NO JK GB Wrote the paper NO JK GB AM.

Chapter 3

Conceived and designed the experiments: JK AM. Performed the experiments: JK PF. Bioinformatics analyses: TM, JK, SK, PF. Analyzed the data: JK TM SK MS. Wrote the paper: JK TM PF MS AM.

Chapter 4

JK conceived the idea and wrote the manuscript. AM supervised and contributed to writing the manuscript.

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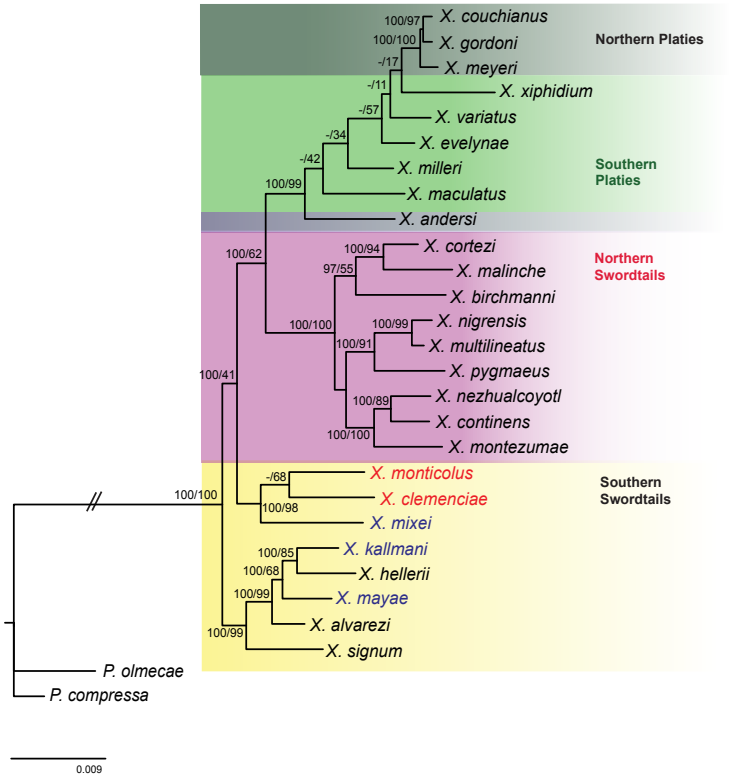
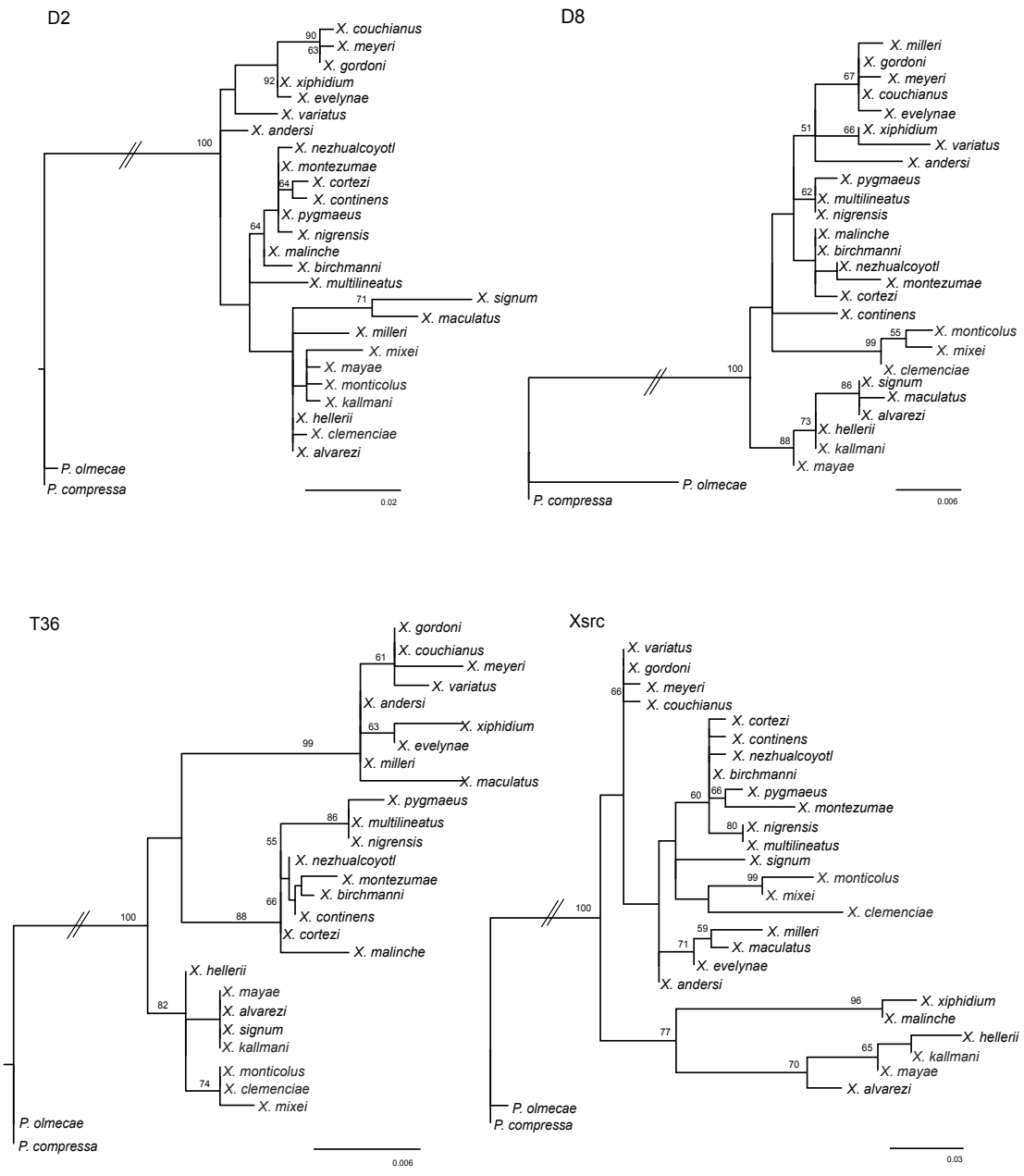
Appendices

Chapter 1

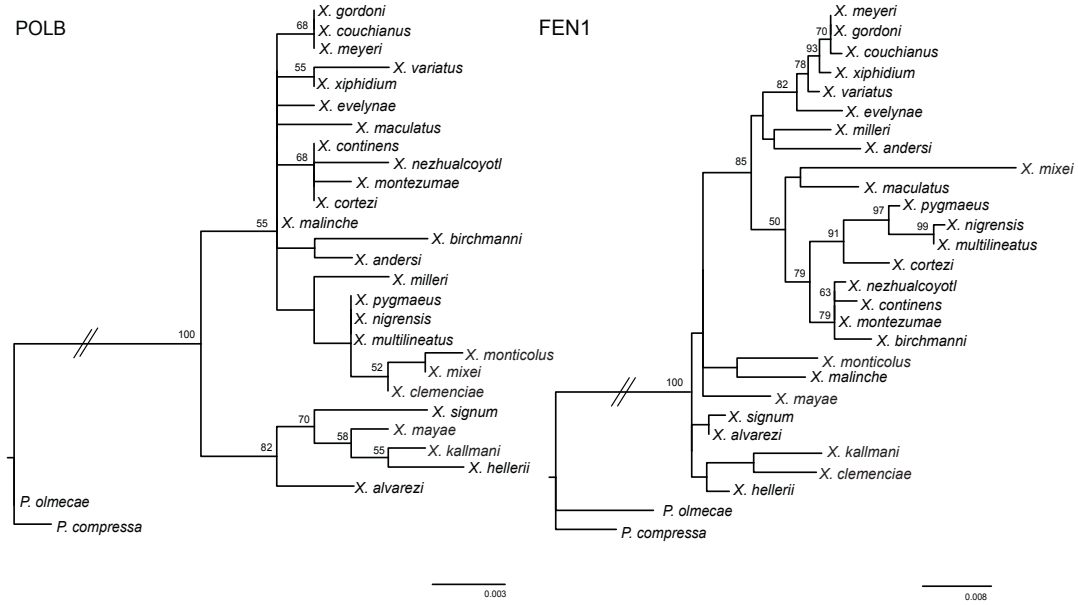
Additional file 1 Combined mitochondrial and nuclear phylogeny of the genus Xiphophorus. The phylogenetic tree was constructed from combined sequences (8515 bp) of two mitochondrial and eleven nuclear loci. Numbers above the nodes indicate Bayesian posterior probabilities and Maximum-Likelihood bootstrap values, respectively.

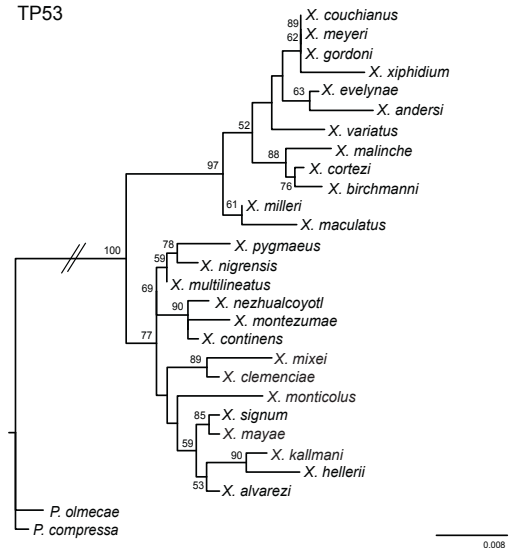


Additional file 2 Maximum-Likelihood trees of the eleven individual nuclear loci (PhyML 3.0). Detailed information for each locus (i.e., evolutionary substitution models) is shown in Table 1.1. Maximum-Likelihood bootstrap values higher than 50 are shown.

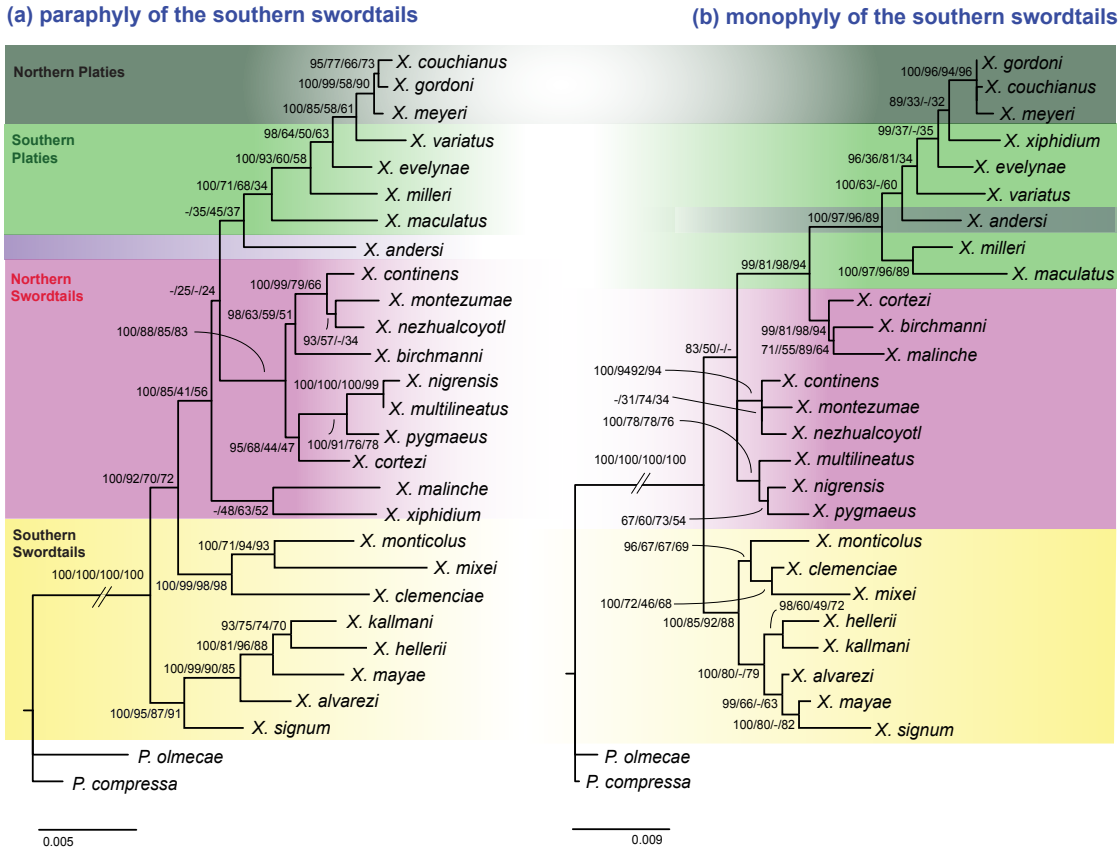


Additional file 2 Continued



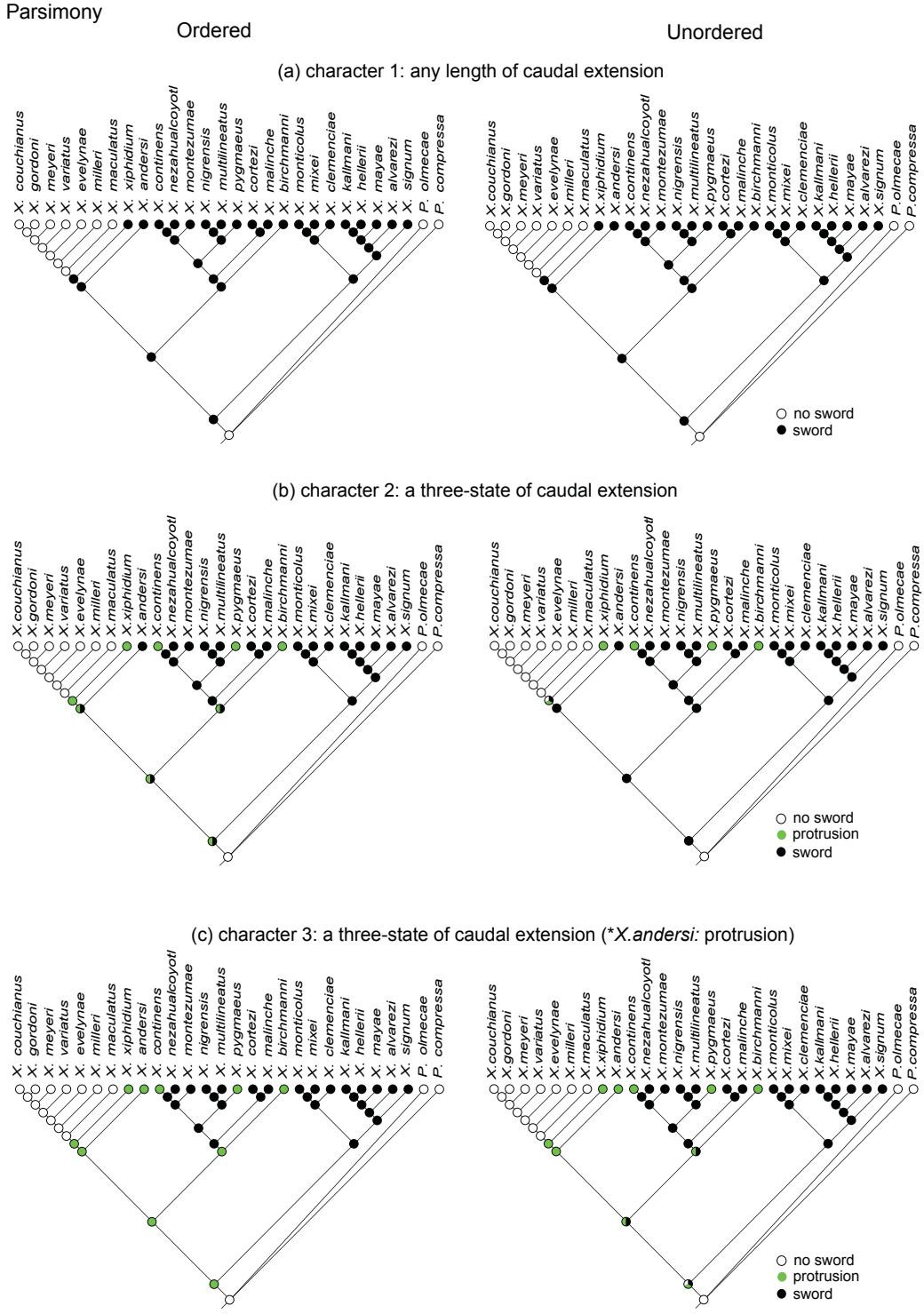


Additional file 3 Nuclear phylogenies based on two sets of nuclear loci suggest monophyly or paraphyly of the southern swordtails. The phylogenetic trees were constructed from (a) combined sequences of seven nuclear loci including D8, X-*src*, Rag1, GNG13, G6PD, POLB and FEN1 (5166 bp) and (b) combined sequences of four nuclear loci including UNG, TP53, T36 and D2 (2110 bp). The full name of these nuclear loci is given in Table 1.1. Numbers above the nodes indicate Bayesian posterior probabilities, Maximum-Likelihood, Neighbor-Joining and Maximum-Parsimony bootstrap values, respectively. The total length of aligned sequences for the first seven loci combined was 5166 bp with 0.015 (SE = 0.001) average nucleotide diversity (*p*-distance) and 267 sites were parsimony informative among 449 variable sites. The second four loci combined included 2110 bp with 0.023 (SE = 0.002) and 145 sites were parsimony informative among 241 variable sites. TVM+G and GTR+G were chosen as the best evolutionary models for the first and second classes of genes, respectively.

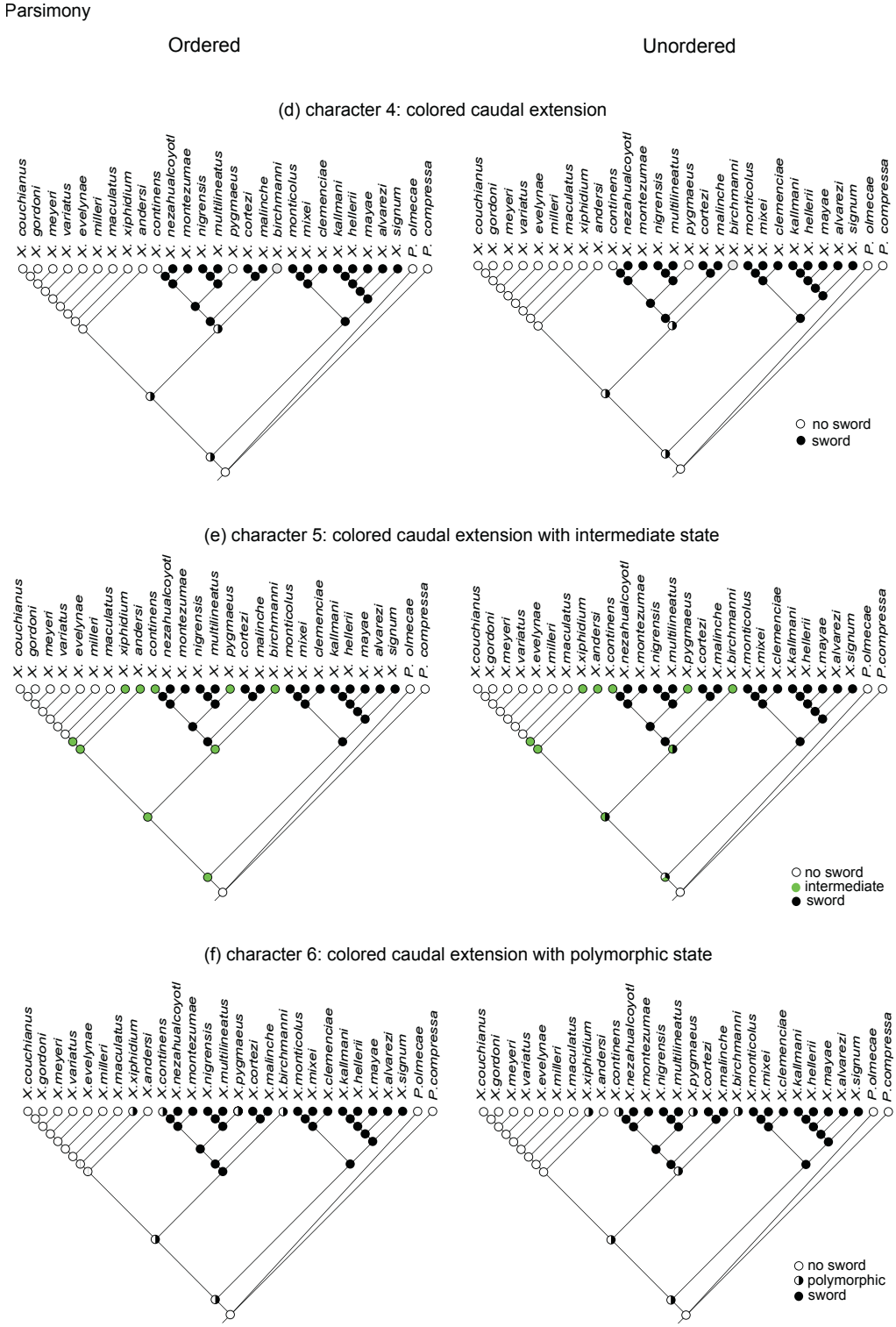


Additional file 4 Parsimony reconstructions for the ancestral state of the sword in the genus *Xiphophorus*. Six different characters were mapped onto a nuclear tree: (a) a two-state character of any length of caudal extension (character 1), (b) a three-state character of sword extension (no sword, protrusion and sword; *X. andersi* was coded as a sworded species, character 2), (c) a three-state character of sword extension (*X. andersi* was coded as a species with protrusion, character 3), (d) a two-state character of colored caudal extension (character 4), (e) a three-state character of colored caudal extension with intermediate state (character 5), and (f) colored caudal extension with polymorphic state (character 6). Each circle on the nodes represents character state (black filled circles: sword; green circles: protrusion (characters 2 and 3) or intermediate within species (characters 5); empty circles: no sword; grey circles: unknown). Transition state was treated as "ordered" (i.e., polymorphic is an intermediated step from 'absent' to 'present' states) or "unordered".

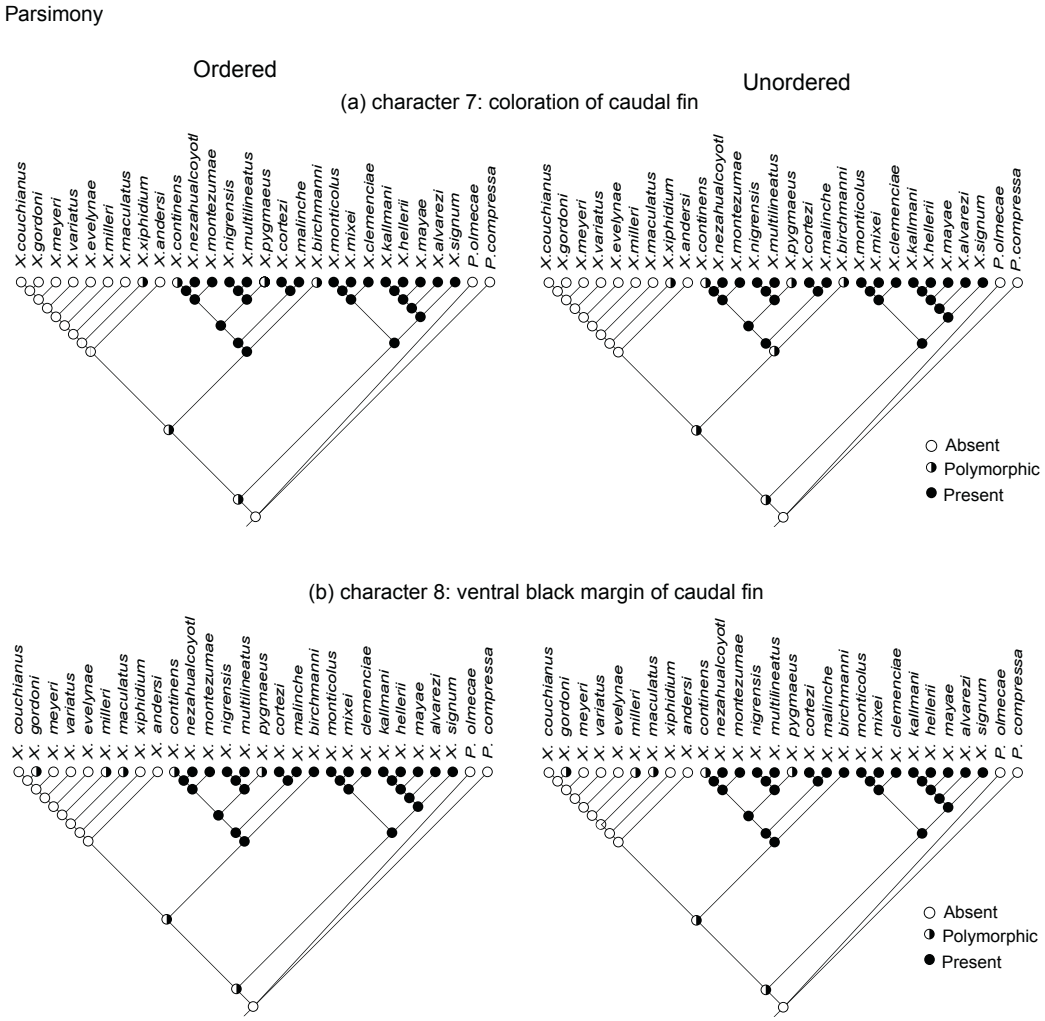
Additional file 4 Continued



Additional file 4 Continued



Additional file 5 Parsimony reconstructions for the ancestral state of (a) coloration (character 7) and (b) ventral black margin (character 8) in caudal fin in the genus *Xiphophorus*. Each circle on the node represents the character state (black filled circles: presence of the character; half black filled circles: polymorphic; empty circles: absence of the character). Transition state was treated as "ordered" (i.e., protrusion is an intermediated step from 'no sword' to 'sword' states) or "unordered".



Additional file 6 Ancestral states of the sword in the genus *Xiphophorus* by maximum-likelihood method. This Table presents the proportional likelihoods for each node of phylogeny.

State	Node	1(a)	2(b)	3, 5 (c)	4 (d)
	-log L	8.284	21.108	21.456	13.437
No sword	All *Xiphophorus*	0.004	0.024	0.040	0.032
Protrusion		-	0.011	0.039	-
Sword		**0.996**	**0.965**	**0.921**	**0.967**
No sword	Platies	0.039	0.004	0.448	**0.949**
Protrusion		-	0.002	0.382	-
Sword		**0.961**	**0.993**	0.170	0.050
No sword	Northern swordtails	0.000	0.025	0.041	-
Protrusion		-	0.049	0.146	-
Sword		**0.999**	**0.926**	0.813	-
No sword	Platies + Northern swordtails	0.007	0.010	0.167	0.124
Protrusion		-	0.032	0.197	-
Sword		**0.993**	**0.867**	0.637	0.875
No sword	Southern swordtails	0.000	0.004	0.008	0.007
Protrusion		-	0.002	0.008	-
Sword		**0.999**	**0.993**	**0.983**	**0.992**

Ancestral state at each node is shown with five different characters: (a) a two-state character of sword extension (character 1), (b) a three-state character of sword extension (no sword, protrusion and sword; *X. andersi* was coded as a sworded species, character 2), (c) a three-state character of sword extension (*X. andersi* was coded as a species with protrusion, character 3) and colored caudal extension with intermediate state (character 5), and (d) a two-state colored caudal extension. The proportional likelihoods for each node are shown and the states judged best according to the threshold as shown in bold.

Additional file 8 Primer information. DNA sequences of newly designed primer-pairs in this study. This Table provides DNA sequences and PCR conditions of newly developed primers.

Name	Locus	Forward	Reverse	Tm (°C)
GNG	Guanine nucleotide binding protein (G protein) gamma 13 (1st intron)	5'-AAGCCTTCAGTATCAGCTG-3'	5'-CGCCTTCTCCACCCAGGGGTT-3'	45.4
G6PD	Glucose-6-phosphate dehydrogenase (6th intron)	5'-CTGGGCAAAGAAATGGTGCA-3'	5'-AGTATCCTCCTCGCCCCTGA-3'	58
UNG	Uracil-DNA-glycosylase (4th intron)	5'-TCACAGCGTCAGTGAAGGTC-3'	5'-AAAGAGCTGGCCACAGACAT-3'	58
POLB	DNA polymerase beta (7th to 11th intron)	5'-CCACCAAAAGATTGGACTCAA-3'	5'-TGGTGTCTCCTTTGGACAGA-3'	52
FEN1	Flap structure-specific endonuclease 1 (3rd intron)	5'-ACCACCTCTGGCTTCAAAAA-3'	5'-TGTATTCTGCTGGGGTGTGA-3'	52
TP53	Tumor protein p53 (4th intron)	5'-GATCCGGAAACTATGCAGGA-3'	5'-CCAATTGGAGTCGTTTTTGC-3'	52

Chapter 3

Supporting Information

Table S3 Functional classifications of sword-specific DEGs.

GO-ID	Term	Category	FDR	P-Value	Fold enrichemnt
GO:0032274	gonadotropin secretion	P	0.012457819	1.71E-06	1.67E+02
GO:0046884	follicle-stimulating hormone secretion	P	0.012457819	1.71E-06	1.67E+02
GO:0050886	endocrine process	P	0.012457819	2.54E-06	4.56E+01
GO:0005576	extracellular region	C	0.038875664	1.06E-05	4.40E+00
GO:0060986	endocrine hormone secretion	P	0.039302941	1.57E-05	7.02E+01
GO:0005788	endoplasmic reticulum lumen	C	0.039302941	2.25E-05	2.54E+01
GO:0032646	regulation of hepatocyte growth factor production	P	0.039302941	2.94E-05	4.45E+02
GO:0032605	hepatocyte growth factor production	P	0.039302941	2.94E-05	4.45E+02
GO:0048178	negative regulation of hepatocyte growth factor biosynthetic process	P	0.039302941	2.94E-05	4.45E+02
GO:0048176	regulation of hepatocyte growth factor biosynthetic process	P	0.039302941	2.94E-05	4.45E+02
GO:0048175	hepatocyte growth factor biosynthetic process	P	0.039302941	2.94E-05	4.45E+02
GO:0060279	positive regulation of ovulation	P	0.047968432	4.90E-05	2.96E+02
GO:0060278	regulation of ovulation	P	0.047968432	4.90E-05	2.96E+02
GO:0032277	negative regulation of gonadotropin secretion	P	0.047968432	4.90E-05	2.96E+02
GO:0046882	negative regulation of follicle-stimulating hormone secretion	P	0.047968432	4.90E-05	2.96E+02

Table S5 Functional annotation clustering for DEGs in the gonopodium (TG) compared non-treated anal fin (CG).

GO-ID	Term	Category	FDR	P-Value	Fold enrichemnt
GO:0005576	extracellular region	C	1.39E-16	9.36E-21	2.40E+00
GO:0044421	extracellular region part	C	2.12E-10	2.84E-14	2.43E+00
GO:0005788	endoplasmic reticulum lumen	C	1.44E-09	2.91E-13	8.52E+00
GO:0031012	extracellular matrix	C	3.35E-06	8.99E-10	2.43E+00
GO:0005578	proteinaceous extracellular matrix	C	8.30E-06	3.90E-09	2.56E+00
GO:0005615	extracellular space	C	2.10E-05	1.13E-08	2.40E+00
GO:0042555	MCM complex	C	3.86E-04	3.32E-07	1.45E+01
GO:0044432	endoplasmic reticulum part	C	5.02E-04	5.06E-07	1.96E+00
GO:0044420	extracellular matrix part	C	1.85E-02	2.98E-05	2.37E+00
GO:0005783	endoplasmic reticulum	C	2.92E-02	5.89E-05	1.53E+00
GO:0005515	protein binding	F	6.48E-06	2.17E-09	1.22E+00
GO:0043565	sequence-specific DNA binding	F	2.75E-02	5.18E-05	1.74E+00
GO:0007275	multicellular organismal development	P	8.30E-06	3.39E-09	1.32E+00
GO:0048731	system development	P	7.00E-05	4.23E-08	1.33E+00
GO:0032502	developmental process	P	9.20E-05	6.18E-08	1.27E+00
GO:0044707	single-multicellular organism process	P	2.48E-04	1.83E-07	1.25E+00
GO:0032501	multicellular organismal process	P	3.86E-04	3.37E-07	1.24E+00
GO:0009888	tissue development	P	4.68E-04	4.40E-07	1.51E+00
GO:0006268	DNA unwinding involved in DNA replication	P	6.99E-04	7.51E-07	2.49E+01
GO:0048856	anatomical structure development	P	2.11E-03	2.41E-06	1.25E+00
GO:0006559	L-phenylalanine catabolic process	P	2.80E-03	3.95E-06	2.66E+01
GO:0006558	L-phenylalanine metabolic process	P	2.80E-03	3.95E-06	2.66E+01
GO:1902222	erythrose 4-phosphate/phosphoenolpyruvate family amino acid catabolic process	P	2.80E-03	3.95E-06	2.66E+01
GO:1902221	erythrose 4-phosphate/phosphoenolpyruvate family amino acid metabolic process	P	2.80E-03	3.95E-06	2.66E+01
GO:0030174	regulation of DNA-dependent DNA replication initiation	P	7.04E-03	1.04E-05	1.01E+01
GO:0048513	organ development	P	1.14E-02	1.76E-05	1.31E+00

GO:0018208	peptidyl-proline modification	P	2.05E-02	3.44E-05	5.28E+00
GO:0048736	appendage development	P	2.05E-02	3.58E-05	2.18E+00
GO:0009074	aromatic amino acid family catabolic process	P	2.59E-02	4.70E-05	1.33E+01
GO:0006271	DNA strand elongation involved in DNA replication	P	2.82E-02	5.48E-05	5.55E+00
GO:0042733	embryonic digit morphogenesis	P	2.99E-02	6.23E-05	4.12E+00
GO:0060173	limb development	P	3.01E-02	6.48E-05	2.43E+00
GO:0022616	DNA strand elongation	P	3.79E-02	8.39E-05	5.22E+00
GO:0048387	negative regulation of retinoic acid receptor signaling pathway	P	4.84E-02	1.11E-04	3.55E+01
GO:0006662	glycerol ether metabolic process	P	4.86E-02	1.14E-04	1.07E+01

Figure S1 Pathway analysis based on the comparison between the sword (TV) and dorsal caudal (TD) fin in the treated fish.

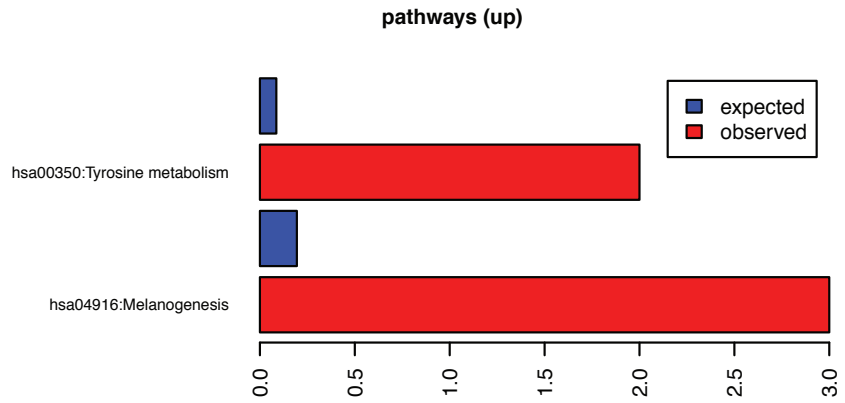


Figure S2 Functional annotation based on the comparison between the sword (TV) and dorsal caudal (TD) fin in the treated fish.

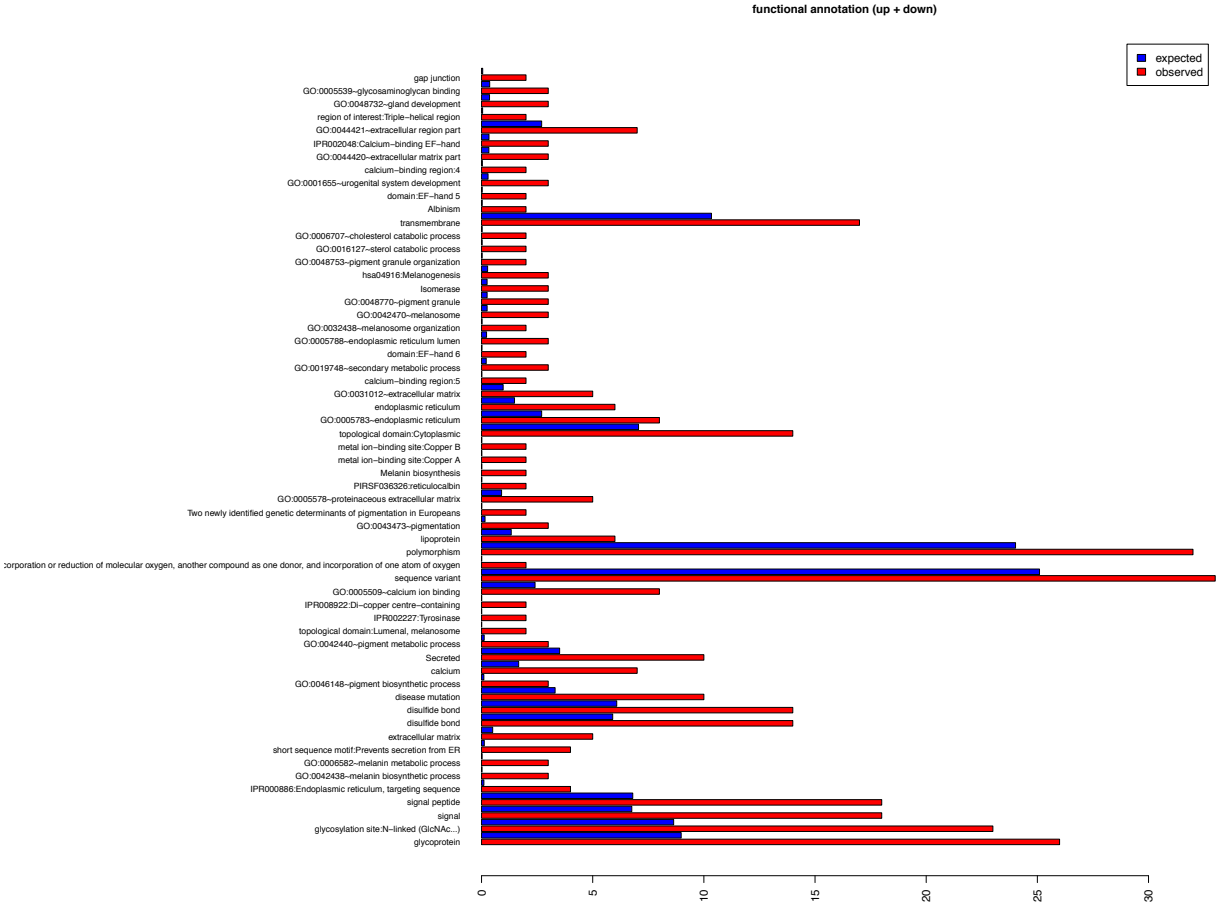


Figure S3 Structure of the gonopodium in adult *Xiphophorus hellerii* (Scale bars: 500 μm) (This figure is modified from (Offen *et al.* 2013))