Title: The developmental and genetic architecture of the sexually selected male ornament of swordtails

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Abstract

Sexual selection results in sex-specific characters like the conspicuously pigmented extension of the ventral tip of the caudal fin - the "sword" - in males of several species of Xiphophorus fishes. To uncover the genetic architecture underlying sword formation and to identify genes that are associated with its development, we characterized the sword transcriptional profile and combined it with genetic mapping approaches. Results showed that the male ornament of swordtails develops from a sexually non-dimorphic prepattern of transcription factors in the caudal fin. Among genes that constitute the exclusive sword transcriptome only two are located in the genomic region associated with this trait; the chaperone, fkbp9, and the potassium channel, kcnh8 that in addition to its neural function performs a role known to affect fin growth. This indicates that during evolution of swordtails a brain gene has been recruited for an additional function in establishing a male ornament.

Introduction

The evolution of male ornaments has intrigued biologists ever since Charles Darwin struggled to explain how exaggerated, expensive and likely deleterious structures like the peacock's tail or the horn of male unicorn beetles might have arisen by natural selection. Twelve years after the publication of his book "On the origin of species", Darwin wrote his second most influential book not on the role of natural, but on sexual selection in evolution [1]. He described the "sword" of the green swordtail, *Xiphophorus hellerii* as an example for his theory on sexual selection and postulated that selection by female choice can be a strong mechanism that could explain the evolution of traits that are clearly detrimental in terms of natural selection [1]. In several species of the genus *Xiphophorus* (Greek for dagger bearer) males carry the sword, a conspicuous extension of the ventral fin rays of the caudal fin that is brightly colored yellow, orange or red and is surrounded by a dark black margin (Fig. 1). The sword develops at puberty and can be as long as the fish itself in some species. Its morphogenesis is instructed by the ventral proximal caudal fin rays, called the "sword organizer" [2]. The sword is a male restricted trait, but female swordtails develop swords like males when treated with testosterone [3, 4]. This suggests that a potential sexual conflict has been solved by a strict androgen dependency for expression of the phenotype. Females of *Xiphophorus hellerii* and several other species preferentially associate with males carrying a longer sword over males with shorter swords, which is thought to result in a higher mating success of long-sworded males [5, 6]. This process exemplifies run-away Fisherian evolution for exaggerated male traits [7]. However, there are also trade-offs [8, 9], because swords attract not only females, but also predators [10], and escape from predators is more difficult because the sword reduces swimming performance [11]. Several species of the genus *Xiphophorus*, including the so-called platyfishes, do not have this sexually dimorphic character (Fig. 1), even though, surprisingly, females nevertheless prefer heterospecific sworded males over their own swordless conspecifics [5]. This observation was used to support a major hypothesis in evolutionary ecology, namely that female

preference may drive sexual selection by sensory exploitation since the bias in females was thought to be older than the sword itself [12, 13]. However, molecular phylogenies showed that the sword is an ancestral state [8, 14-16] and implied that derived swordless species had lost the male ornament secondarily, but retained the presumably ancestral female preference for them. This phylogenetic inference fueled the discussion about which evolutionary forces drove the evolution and loss of this conspicuous trait (see [17, 18] [19-21].

Sword length is a species-specific character and is even polymorphic in two species of Northern swordtails. Females of different *Xiphophorus* species show differences in their preference for sword [5, 22]. Female preferences such as this are considered to potentially not only drive the evolution of male ornaments, but also to result in speciation [23-25]. In the genus *Xiphophorus*, the widespread propensity to prefer sworded males lead to the formation of two hybrid species *X. clemenciae* [8, 21] and *X. monticolus* [16] where, due to the preference for swords females of non-sworded species hybridized with males of swords species to bring about new, sworded hybrid species.

A huge body of literature on how both sexual and natural selection can lead to speciation has been published[26, 27] but almost nothing is known about the genetic basis of male ornaments or male "weapons" used in male-male competition [28, 29]. To identify the genes on which female preferences act on is an important task that is necessary to permit the testing of hypotheses regarding the roles of sexual selection at the molecular genetic level.

The swords of swordtails became a textbook example of a sexually selected trait, yet despite research efforts for almost three decades the molecular genetic basis of sword development remained unkown. So far, candidate gene approaches involving known genes of fish fin growth and development [30] [31] and suppression subtractive hybridization cloning [32] have not revealed the secret of the sword.

To identify the genetic basis for sword formation, we combined genome-wide expression analysis during sword development and regeneration with a genetic association study for sword length in a cross of a non-sworded species to a sworded species.

Results

To obtain a most comprehensive list of protein coding genes that are involved in the formation of the sword, we compared expression levels using several RNA-seq datasets from the green swordtail, *Xiphophorus hellerii* (Fig. 1). We reasoned that sword genes should be differentially expressed (i) during growth of the developing sword of males at puberty (fig. S1) and (ii) during the course of sword regeneration (fig. S2). Because immature fish and adult females also develop a sword indistinguishable from the male structure following treatment with androgens [3, 4] we generated (iii) one RNA-seq dataset from testosterone-treated adult females; and added (iv) our previous dataset from testosterone-induced swords in pre-pubertal juveniles [3]. Small biopsies from the dorsal and ventral fin margin during a timed series of growth and of regeneration and from the hormone induced and naturally

developed swords from 15-20 individuals were pooled and used for transcriptome sequencing. Differential expression was deduced from comparison to the corresponding dorsal part of the caudal fin. The four datasets were overlapped to identify genes that are commonly regulated in all four processes of sword development (fig. S3). This process yielded a set of 68 regulated genes differentially expressed (log2FC >=1) in all sword transcriptomes (11 down and 57 upregulated, table S1).

We expected differentially expressed genes to be of two main categories: those primarily responsible for inducing the sword and those that execute the instruction process by actually building the components of the sword. The sword, like other parts of the caudal fin, consists of bony fin rays, skin, pigment cells, sensory neurons, blood vessels and mesenchyme. Amongst genes upregulated in sword vs control fin regions, four genes (*xdh, tyr, myrip, agrp*) are obviously connected to sword pigmentation; several other upregulated genes are related to increased vascularization (*angptl5, angptl 1)* and fin-ray rigidity *(*collagen*s col9a1, col10a1* and extracellular matrix proteins *fib7l, spock 2, tn-c, frem3, cd200, and4, gpc2*) that support the sword structure as an extremely long outgrowth of ventral fin rays. It is unclear whether these genes are also critical for the primary process of induction and development of the sword, but all are reasonably predicted to be involved in later differentiation processes. The sword transcriptome was also enriched for genes with neural functions (*pdyn*, *draxin*, *kcnh8*, *kcng2*, *chrna7*, *ncan*, *nrxn*, *lypd6, gfra1*) and Ca^{2+} signaling (*stc2*, *efcc1*, *fkbp9*, *fkbp11*).

Intriguingly, several transcription factors were included in the differentially expressed genes list and could be strong candidates for having a critical function in regulating caudal fin development and consequently also sword formation. *Homeobox protein six2a*, which plays a role in chicken hindlimb development [33], forms a continuous dorsoventral expression gradient in the swordtail tail fin (Fig. 2A, table S2), similar to several developmental transcriptional regulators in the establishment of the zebrafish pectoral fin anterior-posterior axis [34]. The dorsalizing factor *zinc finger protein zic1*, which is critical for the development of the homocercal fin shape in fish [35] is highly expressed in the dorsal compartment, but expression is absent from the medial region and all sword transcriptomes (table S2). More strikingly, *homeobox protein hoxb13a*, which is the most caudally expressed *hox* gene in fish [36], has high expression in the non-sword regions of the *X. hellerii* caudal fin, but is not expressed in the sword and the sword-organizer (table S2). During tail fin regeneration, *hoxb13a* is upregulated in the median and dorsal region but not expressed in the outgrowth leading to the sword (Fig. 2). The *t-box transcription factor tbx3a* gene, which promotes formation of the mesoderm cell lineage [37] and is involved in vertebrate limb pattern formation [38], is lowly expressed in the non-sword regions of the tail fin, but abundant in the sword organizer region at the base of the fin, and in the sword during regeneration, natural sword development and hormone-induced sword (Fig. 2, table S2). The same expression pattern is displayed by *paired box protein pax9*, which in fish is a critical factor for development of the hypural plate supporting the peduncle [39], where the caudal fin is inserted (Fig. 2, table S2). Interestingly, *leukocyte tyrosine kinase receptor* (*ltk*)*,* which normally has no spatial expression pattern in the caudal fin of *X. hellerii* males, builds up a local expression pattern in the sword

148 producing blastema similar to that of *hoxb13a* during caudal fin regeneration and natural and hormone
149 induced sword development (fig. S4, table S2).
150 Males of two other swordtail species, *X. montezumae* and *X. monticolus* (fig. S5, 6) showed the same
151 expression gradients and temporal pattern during sword regeneration. Of note, analysis in *X.*
152 *montezumae*, the species with the longest sword (sword index = sword length/standard body length up
153 to 1.9), revealed that the transcription factor expression pattern is immediately initiated in the blastema
154 of the regenerating caudal fin and builds up to the levels of the caudal fin margin and sword during the
155 first days of growth. The platyfish *X. maculatus*, a species which does not develop a sword, and the
156 pygmy swordtail, *X. pygmaeus*, where males have only a tiny unpigmented ventral protrusion of the tail
157 fin but no sword, display the transcription factor gradients in the caudal fin, but these gradients are much
158 less pronounced and at lower transcript levels (fig. S7-9). Phylogenetic evidence suggested that these
159 species have lost the sword secondarily [8, 14]. Apparently, the loss of the male ornamental trait is
160 associated with a decay of this gene expression pre-pattern. The sword arose at the basis of the genus
161 *Xiphophorus* [8, 14]. In, *Priapella*, a swordless sister genus, the tail fin pattern on which the sword is
162 built is already present to a large extent. The expression patterns of *pax9*, *tbx3* and *six2a* are conserved,
163 only *hoxb13a* expression is in additional absent from the dorsal compartment (fig. S8, 9). In the distantly
164 related medaka, *Oryzias latipes*, the tail fin spatial expression patterns of *hoxb13* and *pax9* are like in
165 *Xiphophorus*, however, at much lower transcript levels. However, expression of the medaka orthologs
166 of *tbx3* and *six2a* is not detected in the caudal fin (fig. S9).
167 Importantly, the same expression profile for all five transcription factors was also observed in female
168 swordtail caudal fins (fig. S10, table S1, S2), although at lower expression levels for *six2a*, *tbx3a* and
169 *pax9*. However, this finding indicates that a pre-pattern of transcription factors exists in the caudal fin
170 of both sexes that provides in males the positional information for sword development, but this rules out
171 those genes as candidates for sword induction.
172 Reasoning that genes that are responsible for sword would be expressed only in males, we thus generated
173 transcriptomes from upper and lower terminal caudal fin compartments of females and used these to
174 eliminate genes from candidate status in the sword transcriptome if they showed the same regulation in
175 male and female caudal fin regeneration. This process still left us with 54 candidate genes (table S1).
176 To further reduce the number of genes we performed a genetic mapping approach.
177 Thus, we performed QTL mapping using RAD-tags. Because crossing of a swordtail to a nearest
178 outgroup species prior to evolution of this character (e.g. *Priapella sp.*) is not possible, we used a
179 congeneric species that has lost the sword. A backcross between the sword-less Southern platyfish *X.*
180 *maculatus* and the green swordtail *X.hellerii* using *X.hellerii* as the recurrent parent was generated [40].
181 Mapping the sword-index of 85 backcross males against genetic polymorphisms in the reference
182 swordtail genome revealed significant association with a region on linkage group (LG) 13 (LOD score
183 max likelyhood = 3.86, non-parametric = 4.87) (Fig. 3, fig. S11). A region on LG 1 (LOD score ml =
184 3.17, np = 1.57) and LG 9 (LOD score ml = 2.54, np =2.15) barely failed to reach the significance level.

185 Several minor peaks also appeared on LG's 20 – 24. This result defines the sword as a highly polygenic
186 trait, which is in accordance with the size distribution of sword lengths in platyfish/swordtail hybrids
187 [41].
188 When the positions of sword specific differentially expressed genes (table S1) were examined with
189 respect to the QTL peaks in the 2.0 LOD interval, none of the genes involved in establishing the
190 prepattern and none of the pigmentation, angiogenesis, or ECM genes that were differentially regulated
191 during sword development were found to be encoded in any of the regions identified in the QTL
192 analysis. Only two differentially expressed genes with log2FC >=1 mapped to a QTL peak, both in the
193 main peak on chromosome 13. These are *fkbp9* and *kcnh8*.
194 The gene encoding the chaperone peptidyl-prolyl cis-trans isomerase Fkbp9 is 2- to 3-fold higher
195 expressed in the developing sword than in control tissue and becomes upregulated in sword regeneration
196 at stages 3-4 (fig. S12, table S2). Expression is not elevated in the sword organizer, which weakens its
197 candidacy as a gene responsible for induction of sword development.
198 The other gene that has overlapping candidacy from both gene expression and mapping studies is *kcnh8.*
199 Kcnh8 is a potassium channel of the *ether-à-go-go* (EAG) type that is expressed abundantly in brain
200 and at intermediate levels in ovary and testis (Fig. 4A). Importantly, *kcnh8* is strongly upregulated in
201 the sword during normal development and following androgen treatments, in the sword organizer
202 region, and in the fully developed sword, and becomes strongly upregulated during sword regeneration
203 (Fig. 4B, table S2). It is always amongst the 0.3% of most differentially expressed genes (>21,000 total).
204 Transcripts of *kcnh8* are almost absent from all other fin areas of males and *kcnh8* is only expressed at
205 background levels in female caudal fins.
206 Expression of swordtail Kcnh8 in the Xenopus oocyte system and two-electrode voltage clamp analyses
207 revealed that the protein has the hallmark characteristics of a fully functional voltage gated potassium
208 channel member of the $K_v12.1$ family[42] in terms of voltage activation characteristics, time-dependent
209 activation kinetics, potassium selectivity and inhibition by Ba^{2+} ions (Fig. 5).
210 We found that also *X. montezumae*, which has an even longer sword than *X. hellerii*, has the same high
211 expression of *kcnh8* in the sword and during sword regeneration (fig. S13). Interestingly, in species that
212 develops shorter sword than *X.hellerii* or only tiny protrusions swords, *X. monticolus* and *X. pygmaeus*,
213 *kcnh8* expression during sword regeneration is only weakly upregulated. In the swordless platyfish *X.*
214 *maculatus*, no differential expression of *kcnh8* was noted between the lower and upper compartment
215 and during regeneration of the caudal fin (fig. S13).
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218 **Discussion**
219 Sexually selected traits are present in many species and a hallmark of sexual dimorphism between males
220 and females. The evolutionary mechanism driving their origin, maintenance and role in speciation have
221 been widely studied, but today little is known about the proximate causes, i.e. the genes encoding

222 sexually selected traits and their function in development of the structure, aside a few examples from
223 Drosophila [43, 44]. The sword is a male specific outgrowth of the lower margin of the caudal fin and
224 we wanted to know what genes provoke its sex-specific elongation. The fins of fish are intricate three-
225 dimensional structures composed of numerous cell types. Size, shape, pigmentation and other features
226 of fins are generally highly fixed and specific for different species and certain ontogenetic stages. In
227 many species fins are sexually dimorphic traits [45]. In zebrafish it has been shown that pectoral fins
228 have a regionalized gene expression pattern that creates gradients of transcription factors [34]. We
229 conclude that also in the caudal fin of male swordtails a similar specific regionalized gene activity
230 pattern provides the positional information for development of the sword. The regional expression of
231 the transcription factors Hoxb13a, Six2a, Tbx3a and Pax9 produces a prepattern in the tail fin that is
232 connected to sword development since the expression pattern vanishes in species that have secondarily
233 lost the sword. This pattern is established before the sword develops during puberty and its presence
234 (with minor deviations) in adult females may allow the development of a sword after experimental
235 androgen treatment or as a natural phenomenon in old post-reproductive females [46, 47].
236 To identify those genes that are determining the development of the sword in males we reasoned that
237 such genes should be differentially expressed in sword development and encoded in genomic regions
238 that are linked to this trait. Our QTL analysis, consistent with earlier genetic findings [41], uncovered
239 that several chromosomal regions contribute to the polygenic basis of the male structure. Consistently,
240 the major locus on chromosome 13 fully overlaps a similar broad QTL that was obtained in an
241 independent study for the character sword length in natural hybrids between a swordless (*X. birchmanni*)
242 and a sworded (*X. malinche*) Northern swordtail species[48].We identified two candidate genes that
243 appear to be involved in the development of the sword. Rather than being typical regulators of
244 development and differentiation such as transcription factors or extracellular diffusible growth factors,
245 experiments identified a channel protein, *kcnh8*, and a chaperone, *fkpb9*.
246 In zebrafish long fin mutants, mutations in several potassium channel genes, including *kcnh2a*, *kcnk5b*,
247 and *kcc4a* cause various types of fin overgrowth [49-51]. In fighting fish, *Betta splendens, kcnh8* mis-
248 expression is associated with pectoral fin overgrowth (Wang et al. submitted). A hyperpolarizing
249 mutation in *kcnk5b* causes the long fin phenotype in ornamental goldfish [52]. Mutations disrupting ion
250 channels and ion-dependent signaling are extensively related to abnormal organ development and
251 regeneration via bioelectrical regulation [53]. Potassium channels of the Kcnh family have been
252 implicated in cell proliferation by influencing membrane polarization and thus calcium signaling [54,
253 55]. Increased intracellular calcium levels activate osteoblasts and their precursors [56, 57], which build
254 the fin rays of the overgrowing structures of the long-fin mutants and the *Xiphophorus* sword. Potassium
255 channels can also play a role in cell cycle and proliferation control by mechanisms unrelated to ion
256 channel permeability [55]. Despite this wide spectrum of biological functions of potassium channels
257 besides the classical channel properties, their transcriptional regulation and biochemical interactions are
258 not well understood.

Voltage gated channels of the EAG family are inhibited by intracellular calcium [58]. One function of Fkpb9 besides acting as a prolyl cis-trans isomerase is mediated through its calcium binding Ef-H domain [59]. In zebrafish tailfin growth a predominant role for the calcium activated protein phosphatase calcineurin was shown. In this case inhibition of this pathway led to unscheduled outgrowth of the caudal fin margin [60].

Kcnh8 is the pore forming unit of some voltage-gated potassium channels, which have broad functions mainly in neurotransmitter release and neuronal excitability, but also in epithelial electrolyte transport and cell volume regulation [55, 61]. In zebrafish, due to the presence of duplicate versions of the channel protein coding genes, one paralog obviously can fulfill functions restricted to the fin. Mutations of the "fin" paralog only affect fin growth, while the other channel functions are executed by the second paralog. However, *kcnh8* is present only as a single copy and it is abundantly expressed in the brain and to a lesser extent in the gonads of both sexes and additionally only in the male sword of *Xiphophorus* but importantly not in the corresponding part of the female caudal fin. These expression domains imply that a neuronal gene was recruited during the evolution of the male ornament about 3-5 million years ago, early during the diversification of swordtail fish through a rewiring of its regulatory network rather than by selection on its protein function. The Kcnh8 proteins of *Xiphophorus* species have a few aminoacid changes, which, however, do not correlate with the presence or absence of a sword in males (fig. S14). Thus, it is more likely that the function for sword development has been added to the *kcnh8* gene through changes in gene regulation.

The implication of Kcnh8 activity in natural sword development adds a case of an evolutionary mutant for a potassium channel being involved in regulation of fin growth, which thus far were only seen in laboratory mutants. It appears that the four genes, *kcnh2a*, *kcnk5b*, *kcc4b* and *kcnh8,* govern a common pathway of downstream signaling that connects membrane potential, K+ permeability,eilennummern and calcium homeostasis to the ubiquitous machinery of cell growth and proliferation. Although swordtails, because of their livebearing mode of reproduction are not amenable to transgenic technologies, the induced fin mutants of egg laying fish can be employed to systematically knock-out candidate signal transducers and elucidate the interface between ion channels and growth control.

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293 **Authors contributions**

294 MS, AM and JHP conceived the study and coordinated the work. JA, AA, JC, JW and JHP did
295 the QTL mapping, JO and CS prepared RNA and performed the qRT-PCR experiments, DG
296 and RH characterized the channel properties of *Xiphophorus* Kcnh8, SS and CW analyzed
297 sword growth and regeneration, SK, DK and MGO analyzed the RNA-seq data and intersected
298 the expression with the QTL data, AM contributed RNA-seq data from androgen induced
299 swords, WCW and RW contributed the *Xiphophorus hellerii* genome, MS analyzed all data and
300 drafted the manuscript, all authors were involved in preparing the final version of the
301 manuscript.

302 **Competing interests**

303 All authors declare no competing interests.

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Figures

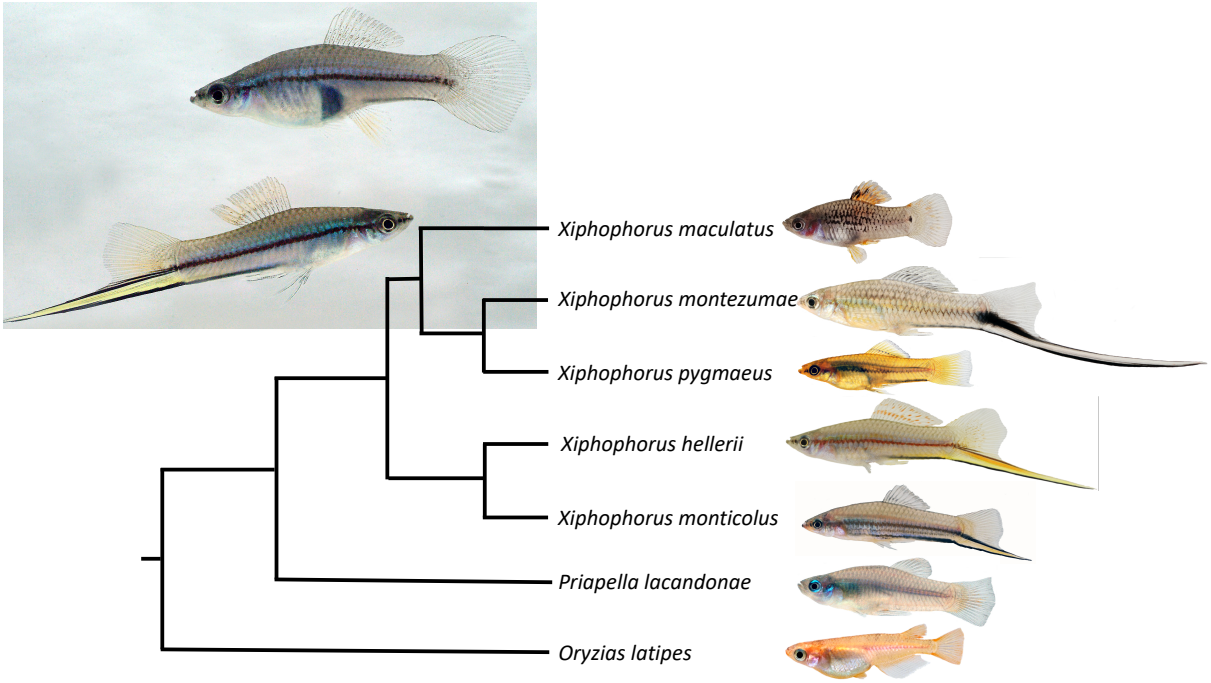
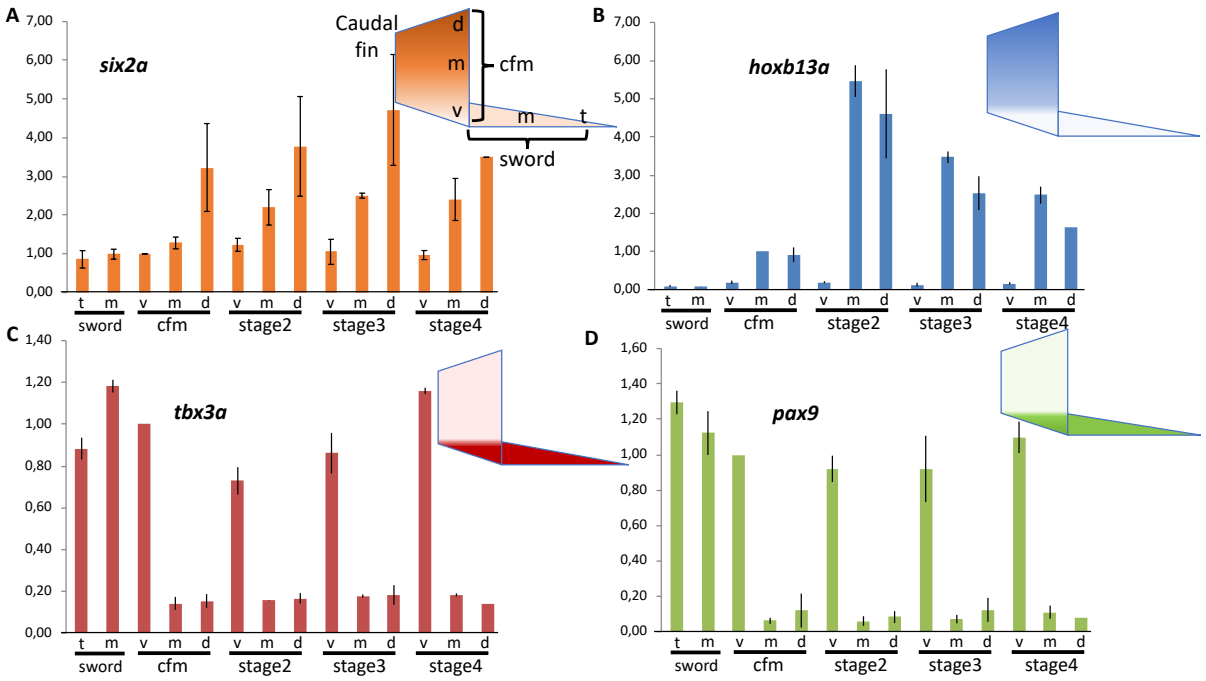


Fig. 1. Phylogenetic relationships of sworded and non-sworded *Xiphophorus* species. The swordless *Priapella lacandonae* is the nearest (sister genus) and medaka, *Oryzias latipes*, a distant outgroup. Insert shows female (upper) and male (lower) of the green swordtail, *Xiphophorus hellerii.*



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468 **Fig. 2. Spatial expression pattern of transcription factor genes in the caudal fin and sword of male**
469 ***Xiphophorus hellerii.*** Expression of *six2a* (A), *hoxb13a* (B), *tbx3a* (C) and *pax9* (D) in the caudal fin
470 margin of the tail fin (cfm) of adult *Xiphophorus hellerii* males, the median sector (m) and tip (t) of the
471 sword and during sword regeneration (v, ventral, m, median, d, dorsal compartment). Vertical axis
472 indicates fold change of expression normalized to cfm, v (*six2a, tbx3a, pax9*) or cfm, m (*hoxb13a*).
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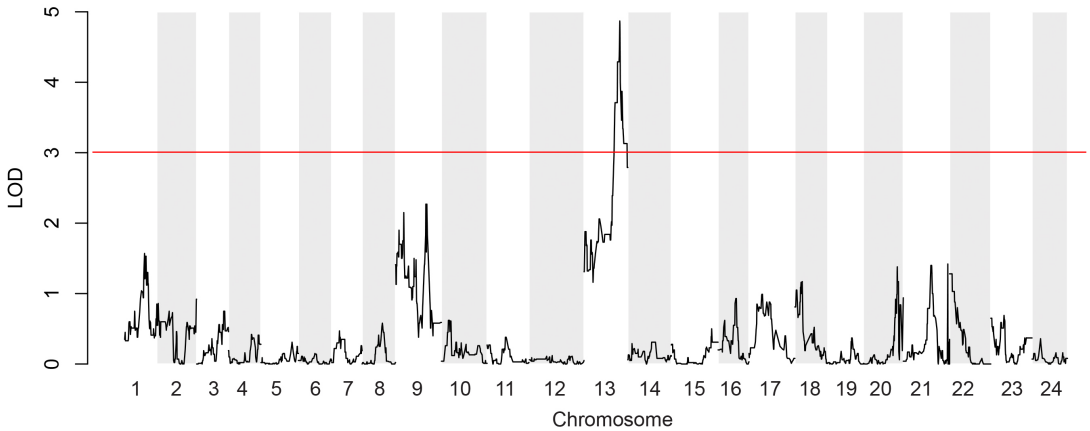


474
475 **Fig. 3. Manhattan plot of quantitative trait loci (QTL) mapping results for sword length.** One
476 major QTL peak is located on chromosome 13, two minor peaks on chromosomes 1 and 9. The plot
477 depicts aligned RAD-tag positions on the *Xiphophorus hellerii* genome version 4.1 with non-parametric
478 statistics
479
480

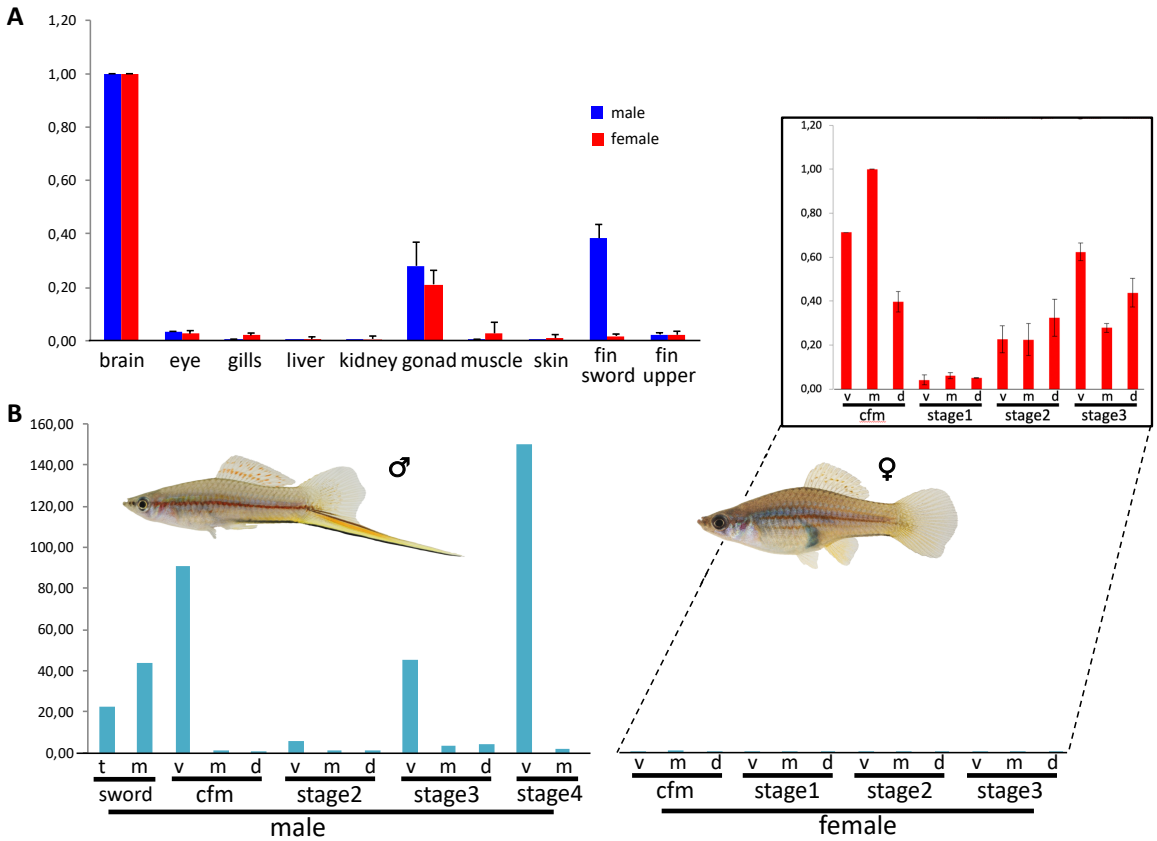
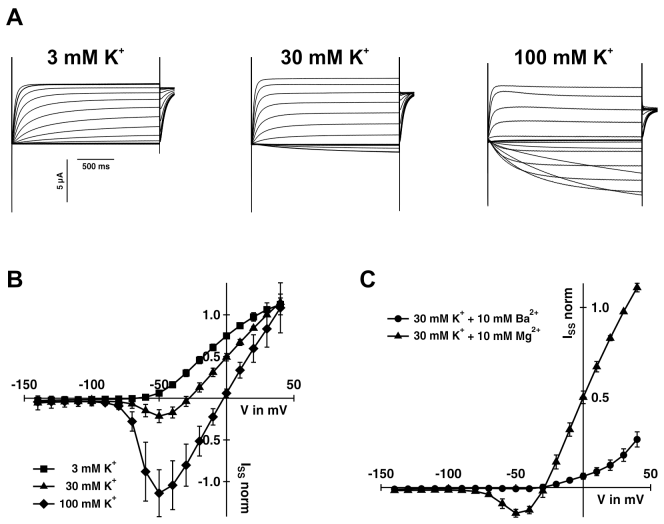


Fig. 4. Expression of *kcnh8* in adult males and females of Xiphophorus hellerii. (A) Organ-specific expression profile in adult females and males. (B) Expression of *kcnh8* in the caudal fin margin of the tail fin (cfm) of adult *Xiphophorus hellerii* males and females, the median sector (m) and tip (t) of the sword and during caudal fin regeneration (v, ventral, m, median, d, dorsal compartment). Insert: expression in females upscaled. Vertical axis indicates fold change of expression normalized to brain (A), cfm, m (B).



490 **Fig. 5 . Electrical features of *Xiphophorus hellerii* Kcnh8.** (A) Representative TEVC recordings of
491 KCNH8-expressing Xenopus oocytes at the indicated potassium concentrations. Test voltages ranged
492 between +40 to -140 mV in 10 mV decrements. (B) Steady-state currents (I_{SS}) extracted from recordings
493 as shown in A) of KCNH8-expressing oocytes were plotted as a function of the applied membrane
494 potential (mean of n ≥ 7 oocytes ± SD of ≥ 3 independent experiments). (C) Application of 10 mM
495 $BaCl_2$ in the presence of 30 mM KCl inhibited the KCNH8-mediated I_{SS} (mean of n = 6 oocytes ± SD
496 of ≥ 2 independent experiments). (B) and (C) I_{SS} were normalized to the currents at +30 mV in standard
497 bath medium (30 mM KCl).
498
499