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ORIGINAL PAPER

Electric organ discharge diversification in mormyrid weakly electric fish is associated with differential expression of voltage-gated ion channel genes

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Abstract In mormyrid weakly electric fish, the electric organ discharge (EOD) is used for species recognition, orientation and prey localization. Produced in the muscle-derived adult electric organ, the EOD exhibits a wide diversity across species in both waveform and duration. While certain defining EOD characteristics can be linked to anatomical features of the electric organ, many factors underlying EOD differentiation are yet unknown. Here, we report the differential expression of 13 Kv1 voltage-gated potassium channel genes, two inwardly rectifying potassium channel genes, two previously studied sodium channel genes and an ATPase pump in two sympatric species of the genus *Campylomormyrus* in both the adult electric organ and skeletal muscle. *Campylomormyrus compressirostris* displays a basal EOD, largely unchanged during development, while *C. tshokwe* has an elongated, putatively derived discharge. We report an upregulation in all Kv1 genes in the electric organ of *Campylomormyrus tshokwe* when compared to both skeletal muscle and *C. compressirostris* electric organ. This pattern of upregulation in a species with a derived EOD form suggests that voltage-gated potassium channels are potentially involved in the diversification of the EOD signal among mormyrid weakly electric fish.

Keywords Weakly electric fish · Ion channels · Electric organ · Gene expression · *Campylomormyrus*

Abbreviations

EOD	Electric organ discharge
gDNA	Genomic DNA
RTqPCR	Quantitative reverse transcription PCR
RIN	RNA integrity number

Introduction

Weakly electric fish are characterized by a specialized electric organ, which produces a species-specific electric organ discharge (EOD) for communication and localization (Lissmann 1958; Moller 1995). To date, at least seven independent origins of an electric organ have been documented among cartilaginous and teleost fish (in Torpedinoids, Rajoids, Mormyroidea, Gymnotiformes, Siluriformes and Uranoscopidae) (Alves-Gomes 2001). Among these, African weakly electric fish of the superfamily Mormyroidea represent one of the largest groups, comprising over 200 species in 21 genera (Alves-Gomes and Hopkins 1997; Feulner et al. 2006; Sullivan et al. 2016).

The electric organ in mormyrids is derived during development from myogenic tissue (Szabo 1960; Bennett 1971; Denizot et al. 1982; Kirschbaum 1983; Bass 1986a). In adult mormyrid species, this electric organ is located in the caudal peduncle and comprised of longitudinally stacked, specialized cells (electrocytes), which generate action potentials (Bennett 1971). The summed activity of

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all electrocytes produces the externally measurable EODs ranging in magnitude from millivolts to a few volts.

The EOD itself is species-specific and often sexually dimorphic, presenting an enormous diversity in signal waveform and duration, which can vary 100-fold across species (e.g., Feulner et al. 2009a). Certain characteristics of the mormyrid EOD, such as number and polarity of phases, can be correlated to electrocyte geometry (Bass 1986b). Several other anatomical features of the electrocytes (i.e., surface proliferation) have additionally been linked to more subtle differences in waveform and duration (Paul et al. 2015). However, not all EOD variation can be explained by the organ's morphology. In fact, the striking variation and rapid evolution of waveform shape and duration among species with similar electric organ anatomy suggests that other core mechanisms are also in play, i.e., the ion currents underlying action potential propagation and EOD production.

In the gymnotiform *Sternopygus*, the use of pharmacological agents and voltage clamps has indicated the involvement of voltage-dependent delayed rectifying and inwardly rectifying potassium channel currents and an inward sodium current in the regulation of electrocyte membrane excitability (Ferrari and Zakon 1993; McAnelly and Zakon 2000; Smith and Zakon 2000). Unfortunately, little data is available on the ionic basis of EOD production in mormyrid fish. However, the sodium channel gene SCN4aa shows evidence for an accelerated rate of evolutionary change in mormyrids compared to non-electric fish (Paul et al. 2016). This is also true for 12 out of 13 shaker-related voltage-gated potassium channel genes (*KCNA*) of the subfamily 1 (Kv1) (Paul, Kirschbaum, Tiedemann, unpubl. results).

During the evolution of vertebrates, teleost fish underwent a whole-genome duplication event (Hoegg et al. 2004; Meyer and Van de Peer 2005). This is reflected in the Kv1 gene family, where 12 of the 13 genes identified in Mormyroidea occur in six paralogous pairs. The large number of duplicated genes may have provided the opportunity for specialization, subfunctionalization or neofunctionalization (Ohno 1970; Steinke et al. 2006). In fact, this has already been suggested for one duplicated voltage-gated sodium channel in weakly electric fish: the gene paralog $Na_v1.4a$ is no longer expressed in the muscle of electric fish, but expressed exclusively in the derived electric organ of several studied gymnotiform and mormyrid species (Zakon et al. 2006).

Here, we chose to investigate the expression pattern of several ion channel genes in two mormyrid species, *Campylomormyrus tshokwe* and *Campylomormyrus compressirostris*. Both species occur in sympatry in the Congo Basin and display biphasic EODs (Feulner et al. 2007; Lamanna et al. 2016). Histological studies have shown that both species possess non-penetrating stalk electrocytes with posterior innervation (Paul et al. 2015). However, despite the similar anatomical organization of their electric organs, the EOD waveform is strikingly different between the two species. The *C. compressirostris* EOD is stereotyped by a short, head-positive peak followed by a second head-negative phase with an average duration of 164 µs. The *C. tshokwe* EOD in contrast, is 22x longer in duration (approximately 3700 µs) due to a prolonged, second head-negative phase nearly twice as long as the initial head-positive phase (Paul et al. 2015) (Fig. 1). Interestingly, both *Campylomormyrus* species studied here show a similar juvenile EOD, comparable in shape and duration to the adult *C. compressirostris* discharge (Nguyen et al. 2017; Fig. 1).

Phylogenetic studies have suggested that short EOD pulses (as observed in *C. compressirostris, C. curvirostris* and *C. tamandua*) may constitute the ancestral type within the *Campylomormyrus* genus (Paul et al. 2015; Lamanna et al. 2016). Elongated and shape-deviated EODs may therefore be a derived form, evolving at least twice in *C. tshokwe, C. rhynchophorus* and *C. numenius* species (Tiedemann et al. 2010; Lamanna et al. 2016). If *C. compressirostris* does display the basal EOD form from which the elongated EOD of *C. tshokwe* has derived, these two species provide an intriguing look into the evolutionary diversification of EOD signals in African weakly electric fish.

We hypothesize that the phenotypic difference in EOD shape and duration between *C. compressirostris* and *C. tshokwe* may result from differential gene expression of ion channels within the electric organ. Using quantitative reverse transcription PCR (RTqPCR), we analyzed the expression patterns of several voltage-gated and inwardly rectifying potassium channel genes, in addition to one ATPase pump and two voltage-gated sodium channel genes, which have been hypothesized to play a role in the evolution of the electric organ and generation of the EOD. In this study, we explore (1) the differential gene expression of these ion channel genes between adult electric organ and skeletal muscle; (2) differential expression in the electric organ between the two species *C. compressirostris* and *C. tshokwe*; and (3) differential gene expression of each *KCNA* paralogous pair in the electric organ.

Materials and methods

Animals

We focused on two species of the mormyrid genus *Campylomormyrus* in this study. Skeletal muscle and electric organ tissue samples were extracted from adult female *C. tshokwe* and *C. compressirostris* individuals. All specimens were captured in the wild in the Congo River near

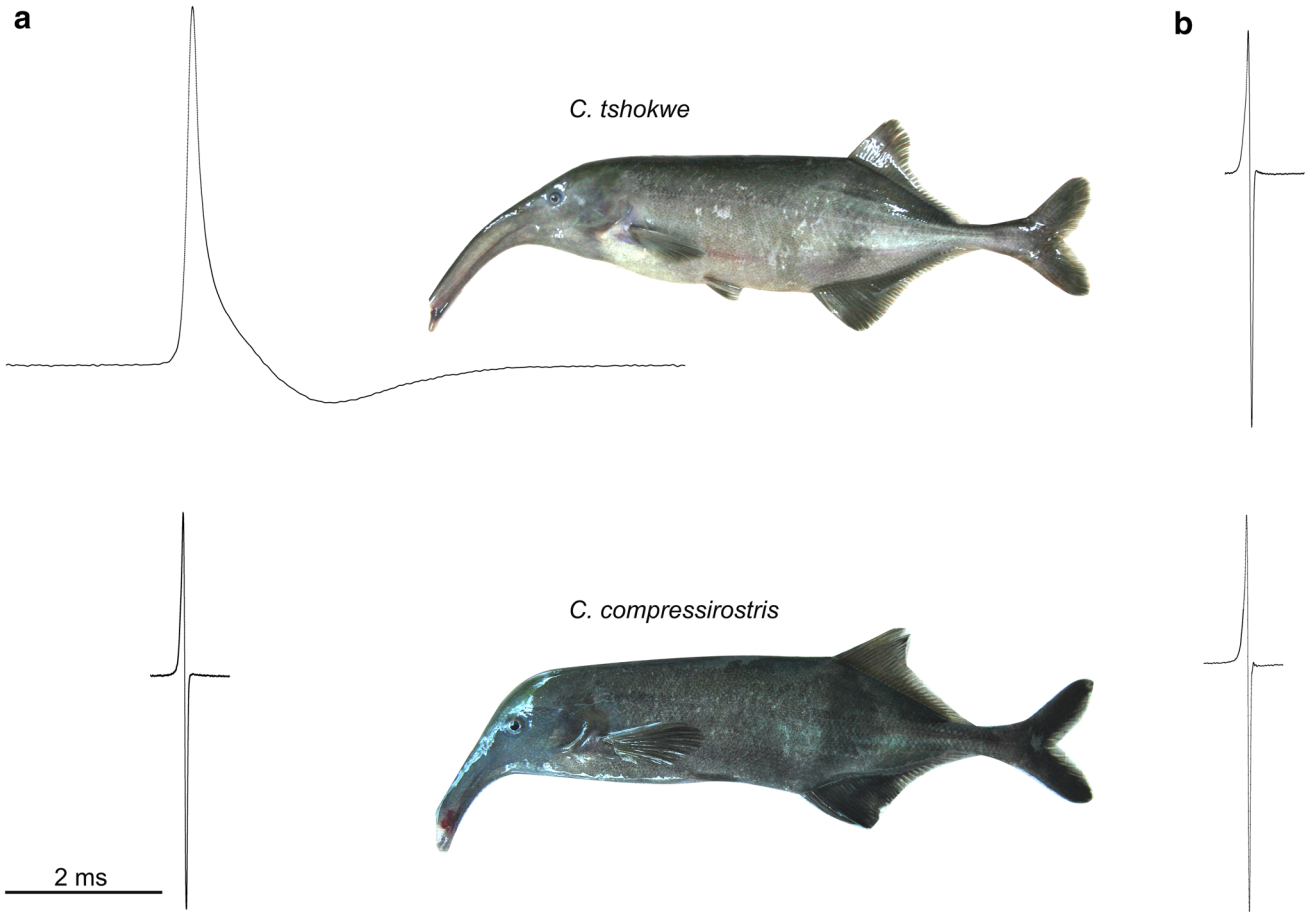

Fig. 1 The two species analyzed in this study *Campylomormyrus tshokwe* and *Campylomormyrus compressirostris* and their respective adult (**a**) and juvenile (**b**) electric organ discharges (EODs). Juvenile EODs were kindly provided by Nguyen et al. (2017)

Brazzaville/Kinshasa. Prior to dissection, they were kept in tanks either at the University of Brazzaville (*C. tshokwe*) or—after import to Germany—at the University of Potsdam (*C. compressirostris*). Three female individuals per species were used in this study (see Supplemental Information for more detailed information). We dissected electric organ (EO) and skeletal muscle tissue samples from the caudal peduncle and the posterior trunk musculature, respectively. Tissue was immediately transferred to vials containing RNA*later* (Life Technologies) and stored at −20 °C until processing.

RNA extraction and reverse transcription

Stored tissue samples were removed from RNA*later*, flash frozen in liquid Nitrogen and homogenized in a β-Mercaptoethanol (β-ME) and RLT buffer solution using a Mini-BeadBeater (Glen Mills Inc.). We extracted total RNA from the electric organ and skeletal muscle using the RNeasy Mini Kit (QIAGEN) and RNeasy Fibrous Tissue Mini Kit (QIAGEN), respectively, according to manufacturer's instructions. To ensure no DNA contamination, samples were treated with DNase I using the RNase-Free DNase Set (QIAGEN).

RNA quality and concentration was determined on an Agilent 2100 BioAnalyzer using the Eukaryote Total RNA Nano Assay. From an electrophoretic trace, the BioAnalyzer system calculates RNA degradation within a sample along a scale of 1 (the most degraded profile) to 10 (the most intact eukaryotic total RNA). This RNA Integrity Number (RIN) ensures a reproducible interpretation of RNA quality (Schroeder et al. 2006). To ensure that we were using high-quality total RNA, only RNA samples with a RIN above 7.0 were considered satisfactory and used for further analysis (Udvardi et al. 2008). Acceptable total RNA samples were diluted to 25 ng/µl and cDNA was synthesized with the oligo$(dT)_{18}$ primer (RevertAid First Strand cDNA Synthesis Kit; Thermo Scientific) according to manufacturer's instructions.

Table 1 RTqPCR primer pairs and amplicon efficiency

Gene	Protein	Primer 5′–3′	Amplicon size (bp)	Mean efficiency ± SEM
Reference genes				
H2A	Histone H2A	F-GAGGTGTTAGAGTTGGCAGGA	159	1.97 ± 0.18
		R-GAGACTTATGGATGTGAGGAATGAC		
elfa	Elongation factor 1-alpha	F-AGGCTGCTGAGATGGGCAAA	121	1.92 ± 0.03
		R-GTACTTGCTGGTCTCAAACTTCC		
rpl13a	L13A ribosomal binding protein	F-TAAGGTTCTGCTGCTTGATGG	101	1.92 ± 0.11
		R-CACCTCACCACCACCACTTT		
Inwardly rectifying potassium channel (KCNJ) genes, subfamily J				
KCNJ1	K_{IR}1.1	F-CCTCTTGCCAGGTGAGGAC	187	1.98 ± 0.15
		R-TTGGTGGTTGTGTGCCTTCT		
KCNJ2	K_{IR}2.1	F-CAACCTACGCAAAAGCCACC	132	1.93 ± 0.06
		R-GTCTACGCCAGTGTCAAAGC		
Voltage-gated sodium channel (SCN) genes				
scn4aa	Na_v1.4a	F-CATGGAGTAAAGACGTGGAGTATG	167	2.10 ± 0.06
		R-CGACAAACTCAGTGATATACGCC		
scn4ab	Na_v1.4b	F-TCGGAAATGCTGACTGTGGG	211	2.04 ± 0.06
		R-AAGACTCGCAACAAACGGAAGG		
ATPase (Na+/K transporting)				
atp1β1a	ATPase, Na+/K+ transporting, beta 1a polypeptide	F-GATTTGAAGACTGTGGAGAAACCC	201	1.99 ± 0.13
		R-ACTTTATACTGGGCATCCTCGG		
Voltage-gated potassium channel (KCNA) genes, subfamily A (*shaker*)				
KCNA1a	Kv1.1a	F-AGAGACTTTGCCCGACTTGA	95	1.94 ± 0.12
		R-GGGTTGAGCAGTATGTTTGGC		
KCNA1b	Kv1.1b	F-TTGACAGAAACCGTCCCAGC	143	2.13 ± 0.23
		R-ACTTCTCCATCGCTTCCACC		
KCNA2A	Kv1.2a	F-CTGCCCATTTTCCGCAATGA	90	1.98 ± 0.11
		R-TGAAGTAGGTTGTGTGATAGCTGG		

Table 1 (continued)

Gene	Protein	Primer 5′–3′	Amplicon size (bp)	Mean efficiency ± SEM
KCNA2B	Kv1.2b	F-TGGTGAGGAGGCCATTGAGA	147	1.97 ± 0.16
		R-GATGGCAATAATCCTAGCAGGC		
KCNA3A	Kv1.3a	F-ACGAGGGCTTCATCAAAGAG	129	1.91 ± 0.02
		R-ACCAGCACCGAGACAATAGC		
KCNA3B	Kv1.3b	F-GACGATAAAGACCTAAGTACCGTGG	97	1.88 ± 0.12
		R-GGTCTCAATAACAAAGAAGGGATCGG		
KCNA4A	Kv1.4a	F-AAAGTTCAAGCCCTGCGAGAG	146	1.95 ± 0.04
		R-TGCCAGGATGTGATGAGTTG		
KCNA4B	Kv1.4b	F-ACTTCATTGGCGCTGGTAGC	98	2.00 ± 0.03
		R-ACCAAATGATGCACACAGTCTC		
KCNA5B	Kv1.5b	F-AGGAGAAACCTTTGCCCCAGA	155	1.98 ± 0.07
		R-AACTCAGGTAACGTCTCCATGC		
KCNA6A	Kv1.6a	F-TTCTTTGCCTGCCCTAGCA	120	2.03 ± 0.06
		R-CGTTCCCTTGATGTTCTGCG		
KCNA6B	Kv1.6b	F-CCGATCCATTCTTTATCGTGGAG	88	1.91 ± 0.09
		R-GGGTTTACTGGGAGACGCAA		
KCNA10A	Kv1.10a	F-CATACGTTTCATTGCCTGTCCC	126	1.94 ± 0.10
		R-ATCCATGTTGGTCGCTATGTCC		
KCNA10B	Kv1.10b	F-TCATCGTGTGTCCCAGCAAA	177	1.95 ± 0.08
		R-AACACCCTGACAAGACGGAC		

Gene expression

We tested commonly used reference genes from the literature for stabile gene expression in skeletal muscle and electric organ in *C. tshokwe* and *C. compressirostris*. Histone *H2A*, elongation factor 1-alpha *elfa*, and L13A ribosomal binding protein *rpl13a* were identified as the most stable and used as endogenous references in further analyses (Hibbeler et al. 2008; McCurley and Callard 2008; Lamanna et al. 2014).

Gene-specific oligonucleotide primers for the 3 reference genes, 13 *KCNA* channel genes (Kv1.1a, Kv1.1b, Kv1.2a, Kv1.2b, Kv1.3a, Kv1.3b, Kv1.4a, Kv1.4b, Kv1.5b, Kv1.6a, Kv1.6b, Kv1.10a, Kv1.10b), two *KCNJ* channel genes ($K_{IR}1.1$, $K_{IR}2.1$), two *scn* genes ($Na_v1.4a$, $Na_v1.4b$) and an ATPase Na+/K + transporting polypeptide (*atp1β1a*) were developed from genomic DNA (gDNA) sequences in *C. tshokwe* and *C. compressirostris* (Table 1). To avoid a fluorescent signal bias based on amplicon length, all amplicons have similar lengths. To guarantee specificity, at least one primer per pair showed ≥30% difference between paralogs. All primers anneal at 60 °C. To exclude the possibility of gDNA contamination, primers either span an exon–exon junction or are found in two consecutive exons of the gene

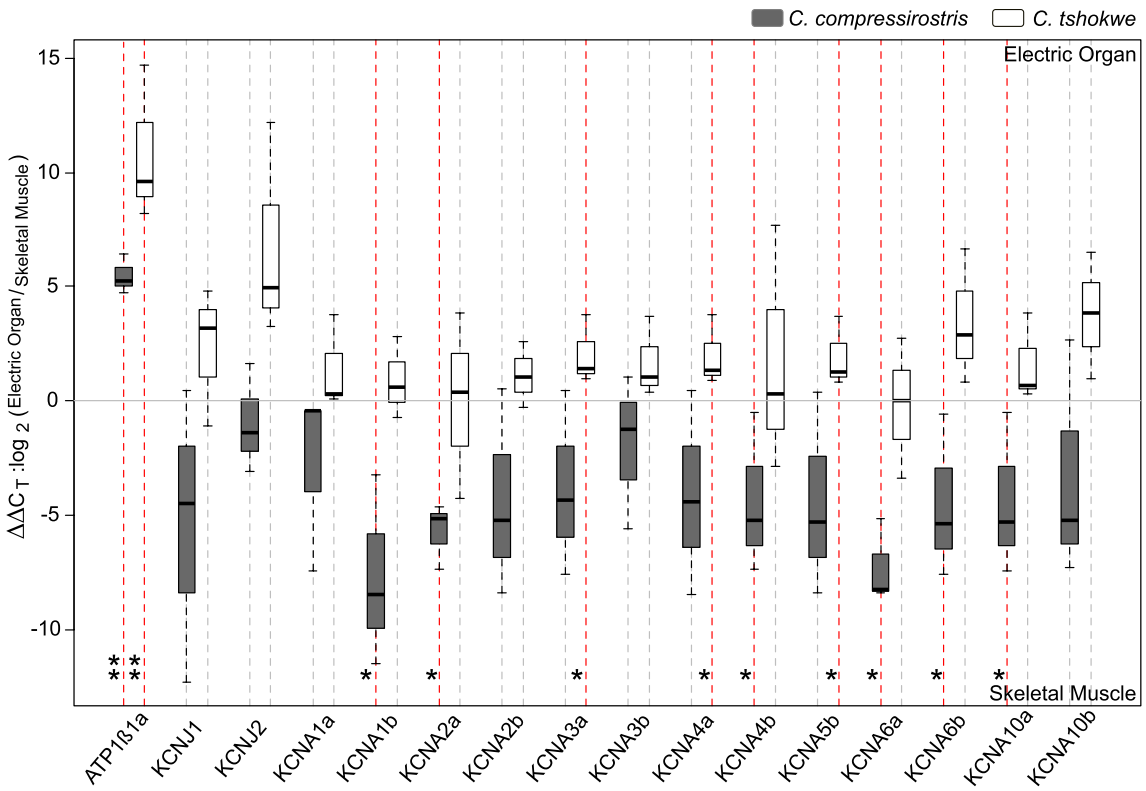

Fig. 2 Differential gene expression (Log_2 relative expression) of the ATPase Na+/K+ ion pump *atp1β1a*, 2 *KCNJ* genes and 13 *KCNA* genes in *C. tshokwe* ($n=3$; *white*) and *C. compressirostris* ($n=3$; *grey*). *Positive values* indicate a higher expression in the electric organ respective to skeletal muscle; *negative values* indicate a higher expression in skeletal muscle. Genes marked with a *red dotted line* have a significantly different expression across tissues [*stars* (*) indicate significance level; Welch's two-sample *t* test]

(exception was made for *KCNJ* and *KCNA* channel genes, which are intron-less). We verified band size and specificity of the primers on cDNA template using gel electrophoresis and Sanger sequencing (Applied Biosystems ABI Prism 3130×1 Genetic Analyser). Sequenced amplicons were then controlled using BLAST to ensure accuracy.

Given that RTqPCR accuracy is dependent on the efficiency of the PCR amplification, we established PCR amplification efficiency for each primer pair from a standard curve of a fivefold dilution series. qPCR reactions were run on a 7500 Fast qPCR system (Applied Biosystems) in MicroAmp Optical 96-well reaction plates (Applied Biosystems). Samples were prepared using 1x Sensi-Mix™ SYBR Low-ROX (Bioline), 100 nM primers and 2 µl cDNA template for a final reaction volume of 20 µl. Thermocycling parameters were as follows: 50 °C for 20 s, a 95 °C holding stage for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. For a final control of primer specificity, a melt curve followed each qPCR run: 95 °C for 15 s, 60 °C for 60 s, 95 °C for 30 s and 60 °C for 15 s. Control reactions without template ("no template controls", NTCs) for each primer pair were included to identify any potential unintended amplification products. Each sample point was run in triplicate. The slope of the log-linear portion of the calibration curve was used to calculate the PCR efficiency according to Bustin et al. (2009). Assuming efficiency $=10^{-1/\text{slope}}$, a theoretical maximum of 2.0 (or 100%) indicates that the amount of product doubles with each cycle. In this study, we considered primer pairs with a mean efficiency between 1.9 and 2.1 across all samples and tissues acceptable (Table 1). This is in accordance with variations reported by other groups (Kaiser et al. 2000; Pfaffl et al. 2002; Budhia et al. 2006).

For the final expression analysis, all genes for each tissue sample were run on a single plate. Three biological replicates per species (and tissue) were analyzed. To avoid technical errors, for each biological replicate, all genes were run in triplicate. Data were generated and visualized using the 7500 Software v2.0.1 (Applied Biosystems).

Statistics

For each tissue sample, we calculated the geometric mean of the three technical replicates and used this for further analysis. Normalized expression levels (ΔC_T) were then calculated using the geometric mean of the three reference

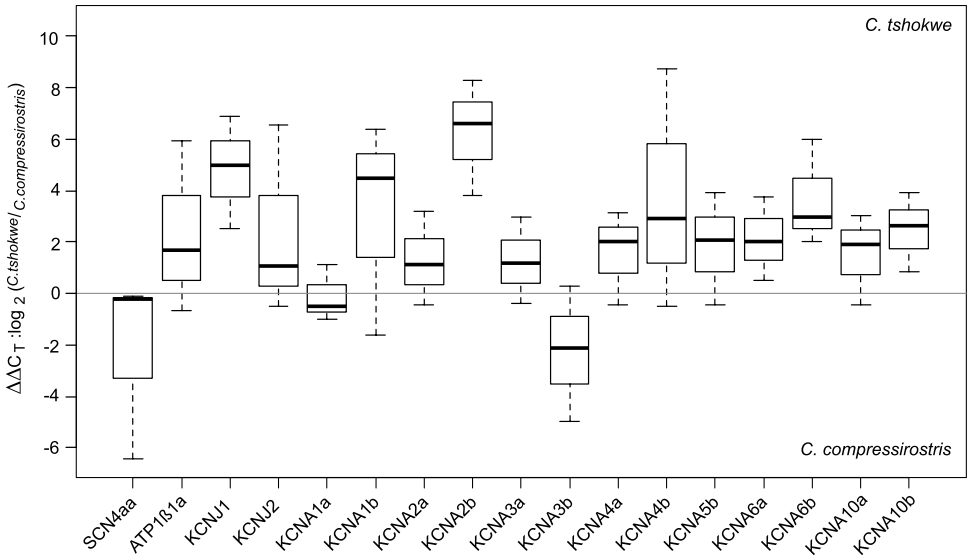

Fig. 3 Differential gene expression (Log_2 relative expression) of a *scn* gene, ATPase Na+/K + ion pump *atp1β1a*, 2 *KCNJ* genes and 13 *KCNA* genes in *C. tshokwe* ($n = 3$) and *C. compressirostris* ($n = 3$). *Positive values* indicate a higher expression in the electric organ of *C. tshokwe* respective to *C. compressirostris; negative values* indicate a higher expression in the electric organ of *C. compressirostris*

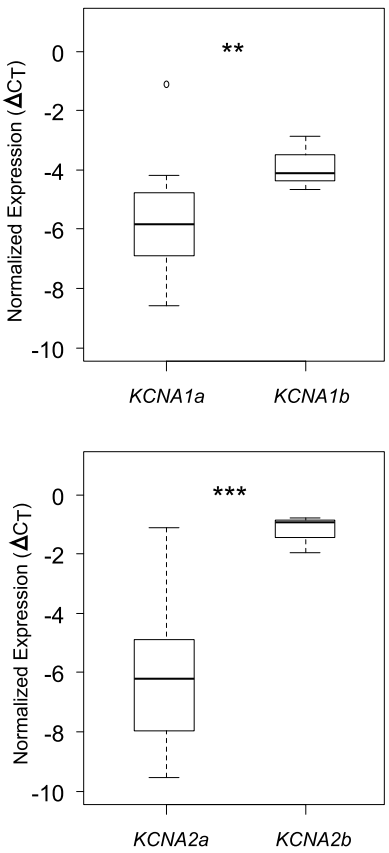

Fig. 4 Normalized gene expression of *KCNA* paralogous pairs in *C. tshokwe* electric organ ($n = 3$). All six gene pairs were tested in this study, but only significant changes in expression across pairs are shown here (*stars* (*) indicate significance; $p < 0.05$; Welch's two-sample *t* test). No gene pairs had a significant change in expression in *C. compressirostris* electric organ (data not shown)

genes *elf1, H2A* and *rpl13a* ($C_{T\ \text{Reference}} - C_{T\ \text{Target}}$). Fold change ($\Delta\Delta C_T$) was calculated as the difference in normalized expression ($\Delta C_{T\ \text{EO}} - \Delta C_{T\ \text{MUSCLE}}$; $\Delta C_{T\ \text{EO.tshokwe}} - \Delta C_{T\ \text{EO.compressirostris}}$). Pairwise differences in expression among genes and tissues were evaluated from the normalized expression levels (ΔC_T) using Welch's two-sample *t* test. All statistical tests were done in R using the integrated development environment RStudio (RStudio 2015; R Development Core Team 2008).

Results

Voltage-gated potassium channel genes, subfamily 1

To determine expression pattern differences of ion channel genes, RTqPCR reactions were performed on tissue extracted from two *Campylomormyrus* species: *C. tshokwe* and *C. compressirostris*. All 13 voltage-gated potassium channel genes present in the mormyrid genus *Campylomormyrus* were expressed in both the adult electric organ and skeletal muscle (Kv1.1a, Kv1.1b, Kv1.2a, Kv1.2b, Kv1.3a, Kv1.3b, Kv1.4a, Kv1.4b, Kv1.5b, Kv1.6a, Kv1.6b, Kv1.10a, and Kv1.10b).

Comparisons between skeletal muscle and adult electric organ show that all *KCNA* genes have a higher expression in the electric organ of *C. tshokwe*, with a significant increase in expression in Kv3a, Kv4a and Kv5b channel genes (p value < 0.05; Welch two-sample *t* test). In contrast, no *KCNA* genes are upregulated in the electric organ of *C. compressirostris*, but several channel genes (Kv1b, Kv2a, Kv4b, Kv6a, Kv6b and Kv10a) show a significantly lower expression in the electric organ compared with skeletal muscle (p value < 0.05; Welch two-sample *t* test) (Fig. 2).

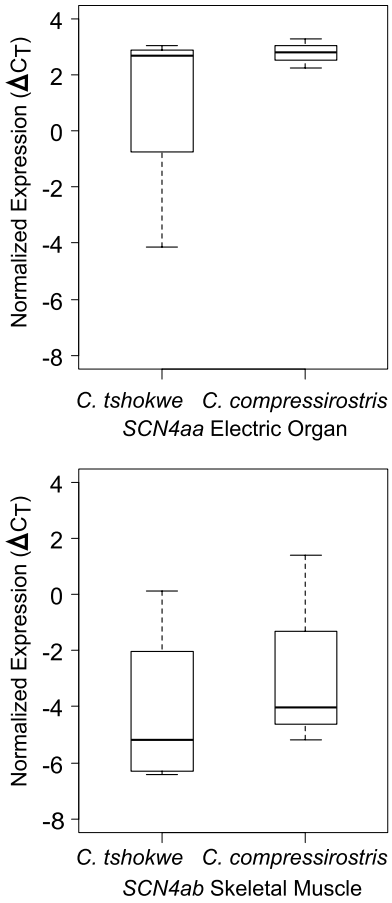

Fig. 5 Normalized gene expression of voltage-gated sodium channel genes (*scn*) in both *C. tshokwe* ($n=3$) and *C. compressirostris* ($n=3$) electric organ and skeletal muscle. There was no significant change in expression of either gene across the two species studied. We found no detectable expression of *scn4aa* in skeletal muscle, nor *scn4ab* in the electric organ of either species

Both species addressed in this study display a biphasic EOD, produced across non-penetrating stalk electrocytes with posterior innervation (Paul et al. 2015). Despite this similar electric organ anatomy, the two species have a significantly different EOD waveform and duration (Fig. 1). We compared *KCNA* gene expression in the electric organ between *C. tshokwe* and *C. compressirostris*. We found that the Kv2b channel gene had a significantly higher expression in the electric organ of *C. tshokwe* compared with *C. compressirostris* (p value <0.05; Welch two-sample t test). In effect, most *KCNA* gene paralogs showed a tendency towards higher expression in *C. tshokwe* electric organ compared to *C. compressirostris* (Fig. 3).

In addition to the differential expression of *KCNA* genes in the electric organ across species, it is possible that variation in EOD form and duration arose from neofunctionalization after a gene duplication event. Since all but one *KCNA* gene (Kv1.5b channel) under examination are expressed in paralogous pairs, we examined the expression differences between these pairs in the electric organ. Here, we found a significantly higher expression of Kv1b and Kv2b channel genes in *C. tshokwe* compared to their respective paralog Kv1a and Kv2a (p value <0.05; Welch two-sample t test) (Fig. 4). In *C. compressirostris*, comparisons among each paralogous pair showed no significant change in expression (data not shown).

Inwardly rectifying potassium channel genes, subfamily J

In addition to the voltage-gated *shaker*-related subfamily A, we were interested in other potassium channels that might contribute to the generation of the EOD waveform. Based on transcriptome data, we choose to investigate two inwardly rectifying potassium channel genes (subfamily J) $K_{IR}1$ (*KCNJ1*) and $K_{IR}2$ (*KCNJ2*) (Lamanna et al. 2014). Similar to *KCNA* expression patterns in *C. tshokwe* and *C. compressirostris*, both $K_{IR}1$ and $K_{IR}2$ genes have a higher expression in the electric organ compared to skeletal muscle in *C. tshokwe* (Fig. 2). Additionally, $K_{IR}1$ displays a significantly higher expression in the electric organ of *C. tshokwe* when compared to *C. compressirostris* (p value <0.05; Welch two-sample t test) (Fig. 3).

Voltage-gated sodium channel genes

In previous studies involving both mormyrid and gymnotiform weakly electric fish, $Na_v1.4a$ (*scn4aa*) has been found to be exclusively expressed in the electric organ (Zakon et al. 2006; Arnegard et al. 2010). Much attention has therefore been given to this gene and its paralog $Na_v1.4b$ (*scn4ab*) for their potential role in the evolution of the electric organ and differentiation of the EOD across species (Zakon et al. 2008, 2009). It has also been hypothesized that the inactivation kinetics of the sodium channel could play a role in the pulse duration of the EOD (Zakon et al. 2006; Paul et al. 2016). We therefore examined the expression pattern of these two widely studied voltage-gated sodium channel genes in *C. tshokwe* and *C. compressirostris* in both the electric organ and skeletal muscle. In both species, we found almost no detectable expression of $Na_v1.4a$ in skeletal muscle nor $Na_v1.4b$ in electric organ, corresponding to previous findings. The $Na_v1.4$ paralogs are similarly expressed in both species (Fig. 5).

ATPase pump

We found a significant upregulation of the Na+/K+transporting beta 1a polypeptide ATPase pump *atp1β1a* in the electric organ compared to skeletal muscle in both species (p value <0.01; Welch two-sample t test) (Fig. 2).

Discussion

A considerable amount of variation seen in EOD waveform, including polarity and number of phases, can be linked to the anatomy of the adult electric organ in mormyrid fish (Bass 1986b; Paul et al. 2015). However, not all differences can be attributed to anatomical variation. In this study, we found a significantly higher expression of the Na+/K+transporting pump *apt1β1a* in the electric organ respective to skeletal muscle in both species studied, *C. compressirostris* and *C. tshokwe*. This correlates with various other studies showing an upregulation of ion pumps and transporters in the electric organ of both mormyrid and gymnotiform species (Gallant et al. 2012, 2014; Lamanna et al. 2014). This is generally expected, given that the summed action potentials across all electrocytes in the electric organ generate the externally measurable EOD (Zakon et al. 1999). Maintaining the electrochemical gradient across the plasma membrane is the major function of ATPases.

This observed upregulation of the Na+/K+transporting pump in electric organ suggests that the concentration of ions and ion channels in the electrocytes, at least in part, influence electric organ function and perhaps EOD diversification. Voltage-gated ion channels in particular are key factors influencing the threshold, waveform and firing pattern of action potentials (for review see Jan and Jan 1997).

Voltage-gated channel genes

Here, we report that all 13 Kv1 voltage-gated potassium channel genes identified in the mormyrid genus *Campylomormyrus* are expressed in both the adult electric organ and skeletal muscle of *C. compressirostris* and *C. tshokwe*. This contrasts with a previous study on the expression pattern of several *KCNA* genes in the gymnotiform *Sternopygus macrurus*, which found no detectable expression of Kv1.1b and Kv1.3 in the electric organ (Few and Zakon 2007). This is an interesting difference, as these two taxa of weakly electric fish evolved their electric organ in convergence. To the best of our knowledge, this is the first study specifically addressing the expression of potassium channel genes in the electric organ in mormyrid species.

In *C. tshokwe*, all *KCNA* genes analyzed have a tendency towards higher normalized gene expression in the electric organ relative to skeletal muscle, with three channel genes (Kv1.3a, Kv1.4a and Kv1.5b) showing a significantly increased expression pattern. Interestingly, none is upregulated in the electric organ of *C. compressirostris* when compared with skeletal muscle expression. This correlates with a previous transcriptome study showing that *C. tshokwe* has a more diverse gene expression pattern than *C. compressirostris*, with a significantly larger percentage of expressed genes being upregulated in the electric organ (59% in *C. tshokwe* compared with 27% in *C. compressirostris*) (Lamanna et al. 2015).

In developmental studies, phenotypic changes can often be attributed to gene expression regulation in both time (when genes are differentially expressed during development) and location (where genes are differentially expressed in the body) (for review see Carroll 2008). The relative increase in *KCNA* gene expression in the electric organ relative to skeletal muscle could therefore have provided a basis for phenotypic diversification of the EOD. However, the relative upregulation in electric organ could also be attributed to a global downregulation of expression in all muscle cells or to a general decrease in the number of muscle cells in which the genes are expressed (Thompson et al. 2014). In addition to cross-tissue comparisons, we therefore also addressed the expression variation of *KCNA* genes in the electric organ between the two species.

Among the 13 *KCNA* genes analyzed, 11 showed a higher relative expression in *C. tshokwe* compared to *C. compressirostris* electric organ. Interestingly, the two voltage-dependent sodium channel genes $Na_v1.4a$ (*scn4aa*) and $Na_v1.4b$ (*scn4ab*) additionally analyzed showed minimal expression variation across the two species in the electric organ and skeletal muscle, respectively.

The sodium channel paralog $Na_v1.4a$ became compartmentalized in the electric organ of at least two independently evolved weakly electric fish lineages (Zakon et al. 2006; Arnegard et al. 2010). Free from constraints, the gene appears to be evolving under positive selection within Mormyridae (Paul et al. 2016), motivating the hypothesis that $Na_v1.4a$ has facilitated the evolution of the electric organ (Ferrari et al. 1995; Markham et al. 2013). The similar expression pattern of this gene in both mormyrid species studied here, which produce considerably different EODs in both waveform and duration, suggests that $Na_v1.4a$ does indeed play an important, underlying role in the production (but not the diversification) of the EOD. In contrast, the comparatively unequal expression of the *KCNA* genes across these two species suggests that these potassium channels may play a role in the evolutionary diversification of EOD signals.

In humans, many physiological properties are dependent on the number and type of ion channels expressed, including action potential duration, frequency and propagation rate (reviewed in Rosati and McKinnon 2004). Fountain et al. (2004) for example, observed a similar upregulation of all but one *KCNA* gene family member in mesenteric resistance artery compared with the conduit of the thoracic aorta; they could show that the quantitative change in *KCNA* gene expression, rather than an all-or-nothing expression pattern, translated partially to physiological function. While we recognize the difficulty in comparing

potassium channels expressed in mammals with those in fish, some studies have indicated pharmacological similarities across taxa (Salkoff et al. 1992). Here, we find an upregulation of potassium channel genes in *C. tshokwe*, which EOD form is considered to be derived within the genus *Campylomormyrus* (Paul et al. 2015; Lamanna et al. 2016). We, therefore, state with caution that this might have physiological implications. Assuming that the expression level of total RNA correlates to the abundance of translated channel protein, we speculate that an upregulation of *KCNA* genes influences diversification of the EOD.

It has been previously reported that in tissues characterized by increased rates of adaptation (such as testis in mammals, or here, in the electric organ) expression patterns tend to reflect phenotypic similarity (Brawand et al. 2011). It would, therefore, be very interesting to analyze the gene expression levels of additional *Campylomormyrus* species with varying EOD waveforms and durations. The expression patterns of *KCNA* genes in the electric organ of juvenile *C. tshokwe* would be of particular interest. Juvenile *C. tshokwe* produces a short EOD waveform similar in duration and form to that of *C. compressirostris* (Nguyen et al. 2017; see Fig. 1). If the upregulation of *KCNA* genes does indeed correlate with signal form, one may hypothesize that juvenile *C. tshokwe* individuals should display an expression pattern similar to *C. compressirostris*, not adult *C. tshokwe*. The expression of potassium channel genes would then increase as the fish developed into an adult.

Given the successful breeding experiments of several captive mormyrid species (Kirschbaum and Schugardt 1995, 2002; Schugardt and Kirschbaum 1998, 2004; Nguyen et al. 2017), it is feasible for future research to focus on ontogenetic differences in gene expression between electric organ and skeletal muscle. RNA-seq could be used for large-scale quantification of RNA, while RTqPCR would provide a finer analysis of gene expression patterns. Both techniques would bolster current studies on the ontogeny of electric organ histology and corresponding phenotypic changes in the EOD (Kirschbaum 1977; Westby and Kirschbaum 1977; Denizot et al. 1982, 1998; Paul et al. 2015; Nguyen et al. 2017). In particular, the up- or downregulation of certain genes in the electric organ as the EOD transitions from juvenile to adult may provide unique insight into those mechanisms underlying EOD diversification.

KCNA gene paralogs

The evolution of novel traits or phenotypes has often been linked to gene duplication events, which have been suspected to relax evolutionary constraints on certain genes (Ohno 1970). Here, 12 of the 13 *KCNA* genes (excluding *KCNA5b*) under investigation are found in six paralogous pairs, likely duplicated during the fish-specific whole-genome duplication event. Although all 13 *KCNA* genes identified here are still expressed in skeletal muscle tissue, one copy of each pair of gene paralogs may have evolved under relaxed selection pressures and/or neofunctionalization. In fact, a study on voltage-gated potassium channel genes in *Campylomormyrus* suggests that several *KCNA* genes have evolved under positive selection in electric fish (Paul, Kirschbaum, Tiedemann, unpubl. results). If one *KCNA* paralog in weakly electric fish has evolved a novel role in EOD production, we would expect it to display a higher expression pattern in electric organ compared to its paralog.

Here, we found two channels, Kv1.1b and Kv1.2b, with a relative increase in gene expression in the electric organ compared to their paralog Kv1.1a and Kv1.2a in *C. tshokwe*, respectively. In *C. compressirostris*, no significant change in expression between paralogous pairs was identified. Given the upregulation of the Kv1.2b channel gene in *C. tshokwe* relative to both its paralog and *C. compressirostris*, it may play a role in shaping the elongated, putatively derived EOD form. We suggest that higher concentrations of Kv1.2b channels on the posterior face of the electrocyte may yield faster kinetics, creating the extremely steep and short head positive phase observed in *C. tshokwe*. While the abundance of Kv1.2b channels could be decisively evaluated with immunoprecipitation, a functional proof of this hypothesis might not be currently feasible in our non-model species.

In the convergently evolved electric organ of South American gymnotiform fish, the differential expression of Kv1.2 and Kv1.1 channel paralogs has also been correlated with phenotypic differences in EOD (Few and Zakon 2007). Interestingly, in gymnotiforms, it appears to be the Kv1.2a paralog (and not the Kv1.2b paralog, as in our species) with a higher expression in electric organs characterized by a high frequency EOD.

Inwardly rectifying channel genes

In addition to voltage-gated channel genes, we were interested in other potassium channels that might play a role in the formation of the EOD. In our expression analysis, $K_{IR}1.1$ (*KCNJ1*) was upregulated in *C. tshokwe* electric organ respective to *C. compressirostris*, but did not show a significantly higher expression compared to skeletal muscle. In zebrafish *Danio rerio*, $K_{IR}1.1$ is expressed in cells associated with osmoregulation (Abbas et al. 2011). $K_{IR}2.1$ (*KCNJ2*), which showed no differential expression, is crucial in determining the duration of the action potential in excitable cells in humans (Pattnaik et al. 2012).

Nonetheless, voltage clamps in the gymnotiform *Sternopygus* indicated the involvement of inwardly rectifying

channel currents in the regulation of electrocyte membrane excitability (Ferrari and Zakon 1993) and transcriptome data in mormyrid species indicate differential expression of several other *KCNJ* genes (i.e., *KCNJ11*) (Lamanna et al. 2015). It would therefore be beneficial to investigate these channel genes in more detail to determine their effect on various EOD phenotypes.

Conclusions

Diversification of the EOD is a likely driver behind the astonishing evolutionary radiation observed in mormyrid weakly electric fish. The species-specific discharge form has been linked not only to niche specialization and foraging, promoting ecological speciation, but may also function in mate recognition, promoting pre-zygotic isolation among sympatric species (Feulner et al. 2009b). The elongated EOD observed in *C. tshokwe*, for example, may be adaptive for prey capture and species recognition (reviewed in Tiedemann et al. 2010). Here, we present evidence that these selective pressures involved in the diversification of the EOD are underlined by the differential expression of voltage-gated potassium channels.

Potassium channels play a crucial role in the regulation of cell membrane potential and are involved in shaping action potential waveforms and modulating the frequency of action potentials (reviewed in Salkoff et al. 1992). We report the differential expression of 13 Kv1 voltage-gated potassium channel genes, 2 K_{IR} inwardly rectifying potassium channel genes, 2 extensively studied voltage-gated sodium channel genes and an N+/K+ ATPase ion pump in two sympatric species of weakly electric fish, *C. compressirostris* and *C. tshokwe*, in both the adult electric organ and skeletal muscle. In doing so, we have identified a potential pattern of upregulation of potassium channel genes in weakly electric fish with elongated, putatively derived EOD forms. This suggests that potassium channel genes are potentially involved in the diversification of the EOD signal and perhaps speciation among weakly electric fish.

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