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Research paper

Characterization of *vasa* homolog in a neotropical catfish, Jundiá (*Rhamdia quelen*): Molecular cloning and expression analysis during embryonic and larval development

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ABSTRACT

We have characterized the full-length *vasa* cDNA from Jundiá, *Rhamdia quelen* (Heptapteridae, Siluriformes). *vasa* encodes a member of the DEAD-box protein family of ATP-dependent RNA helicases. This protein is highly conserved among different organisms and its role is associated with RNA metabolism. In the majority of the investigated species, *vasa* is restricted to the germ cell lineage and its expression has been used to study germline development in many organisms, including fish. The deduced *R. quelen vasa* amino acid sequence displayed high similarity with Vasa protein sequences from other organisms, and did not cluster with PL10 or P68 DEAD-box protein subfamilies. We also reported that there is no other isoform for *vasa* mRNA in *R. quelen* gonads. Expression analysis by RT-PCR and qPCR showed *vasa* transcripts exclusively expressed in the germ cells of *R. quelen* gonads. *R. quelen vasa* mRNA was maternally inherited, and was detected in the migrating primordial germ cells (PGCs) until 264 h post-fertilization during embryonic and larval development. This work has characterized for the first time the full-length *R. quelen vasa* cDNA, and describes its expression patterns during *R. quelen* embryonic and larval development. Our results will contribute to the basic reproductive biology of this native species, and will support studies using *vasa* as a germ cell marker in different biotechnological studies, such as germ cell transplantation.

1. Introduction

vasa encodes a member of the DEAD (Asp-Glu-Ala-Asp) box protein family of ATP-dependent RNA helicase (Linder et al., 1989; Rocak and Linder, 2004). DEAD-box proteins comprised three subfamilies: VASA, PL10, and P68. The *vasa* gene was thought to arise from the duplication of a PL10-related gene prior to the appearance of sponges, but following the diversion of fungi and plants (Mochizuki et al., 2001). It belongs to a complex of RNAs and proteins required for primordial germ cell (PGC) specification, and was first identified in the germ plasm of *Drosophila* eggs (see Nakamura and Seydoux, 2008). Among the transcripts involved in PGC specification, *vasa* is one of the most conserved across evolution, and consequently the most studied universal marker and regulator of germ cell development (Lasko and Ashburner, 1988; Ikenishi and Tanaka, 1997; Kuznicki et al., 2000; Tsunekawa et al., 2000; Hickford et al., 2011; Hartung et al., 2014; Reitzel et al., 2016). In this context, *vasa* homologs have been characterized in many organisms as a specific germ line transcript (Saffman and Lasko, 1999; Raz, 2003), such as in fish, *e.g. Danio rerio* (Yoon et al., 1997), *Dicentrarchus labrax* (Blazquez et al., 2011), *Gadus morhua* L. (Presslauer et al., 2012), *Salmo salar* (Nagasawa et al., 2013), *Carassius auratus gibelio* (Xu et al., 2005), *Clarias gariepinus* (Raghuveer and Senthilkumaran, 2010), *Apostichopus japonicus* (Yan et al., 2013) and *Lates calcarifer* (Xu et al., 2014). Moreover, recent studies have shown that *vasa* is crucial for germ cell development. In invertebrates, *D. melanogaster* females with *vasa (vas)* alleles mutation showed different

Abbreviations: ATP-dependent, adenosine triphosphate-dependent; cDNA, complementary DNA; qPCR, real-time, quantitative polymerase chain reaction; *Rqvasa*, *Rhamdia quelen vasa*; PGCs, primordial germ cells; GFP, green-fluorescent protein; DNAse, deoxyribonuclease; PVC, polyvinyl chloride; hpf, hours post-fertilization; β-actin, beta actin; PFA, paraformaldehyde; PBS, phosphate-buffered saline; mRNA, messenger RNA; rRNA, ribosomal RNA; bp, base pair; UTR, untranslated region; NJ, Neighbor joining; WISH, whole mount *in situ* hybridization

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defects on oogenesis and infertility (Schupbach and Wieschaus, 1991). In mice, targeted mutation of *vasa* (*Mvh)* caused reproductive deficiency in proliferation and differentiation of male germ cell (Tanaka et al., 2000). In the teleost fish medaka (*Oryzias latipes*), *vasa* knockdown led to defects in PGC migration but did not alter their number, identity, proliferation and motility (Li et al., 2009). In zebrafish, disruption of *vasa* resulted in sterility, but only for males (Hartung et al., 2014).

Recent studies have established transgenic lines that express fluorescent proteins under the control of the *vasa* promoter (Krovel and Olsen, 2002; Filby et al., 2014), making it possible to easily monitor PGCs during development (Krovel and Olsen, 2002). These lines are also useful to isolate pure populations of PGCs and germ cells by fluorescence-activated cell sorting for different applications, such as germ cell transplantation. In fish, for example, *vasa::egpf* zebrafish (enhanced green fluorescent protein under *vasa* promoter) was used to isolate spermatogonial stem cell population prior germ cell transplantation (Nobrega et al., 2010; Tonelli et al., 2017).

Germ cell transplantation is a powerful technique that consists of isolating germ cells (PGCs or spermatogonia) from a donor fish and transplanting them into larvae of recipient species during early gonad development (Takeuchi et al., 2003; Okutsu et al., 2006). After transplantation, donor germ cells migrate towards the gonadal ridge and colonize the available niches, originating donor-derived gametes in the adults. Therefore, transplanted animals can function as surrogate broodstock parents by producing offspring with donor genetic characteristics (Takeuchi et al., 2003; Takeuchi et al., 2004; Okutsu et al., 2006). This technique has been successfully described in fish, including salmonids, tilapia, zebrafish and marine species (see review in Lacerda et al., 2010; Nobrega et al., 2010). However, to establish this technique, characterization of early gonadal development in fish larvae is considered one of the most important prerequisites. Molecular markers to identify PGCs in association with morphological analysis would provide a useful tool for characterizing early gonadal development in fish larvae. Moreover, the use of molecular markers for identifying early germ cells would also be applied for selecting PGCs or spermatogonia before transplantation (Takeuchi et al., 2003; Takeuchi et al., 2004; Okutsu et al., 2006; Nobrega et al., 2010).

Considering *vasa* as the most commonly used molecular marker for the germ cell line, and Jundiá, *Rhamdia quelen* (Heptapteridae, Siluriformes) as a potential model for germ cell transplantation as reported by Silva et al. (2016), this work aimed to clone and characterize the full-length *R. quelen vasa* cDNA. After cloning, we examined *vasa* expression during embryonic and larval development by real-rime, quantitative PCR (qPCR) and *in situ* hybridization, showing the usefulness of *vasa* as a molecular marker of PGCs. We also observed PGC migration towards the developing gonadal ridge, which we consider as crucial information to establish the germ cell transplantation technique in this species. In summary, we characterized *vasa* in a neotropical species with great economical importance in aquaculture and showed that *vasa* could be used as a germ cell marker for biotechnological studies in this species.

2. Material and methods

2.1. Ethics statement

All experimental procedures followed the guidelines for the ethical animal treatment and were approved by the Ethics Committee for animal experimentation of the São Paulo State University (protocol number 02248/14). Using approved anesthetics, all efforts were made to minimize discomfort and suffering during experimental procedures.

2.2. Animal stocks and sampling

Jundiá (*R. quelen*, Heptapteridae, Siluriformes) is a neotropical species with a wide distribution from central Argentina until south Mexico (Silfvergrip, 1996). It is considered one of the most promising native species for intensive fish farming due to its great economic interest within freshwater fish cultures, elevated commercial acceptance, elevated sperm production and short sexual maturation (6–8 months) (Ghiraldelli et al., 2007).

All specimens used in this study were reared at Institute for Research in Environmental Aquaculture (InPAA), PR Brazil. Jundiás (*R. quelen*) were kept in freshwater tanks of 200 m^2 under ambient photothermal conditions. Broodstock males and females were used for artificially induced spawning in order to produce eggs and sperm. The eggs were distributed into a conical PVC incubator of 200 l of volume at 22 °C. Embryos and larvae at different developmental stages were collected: at 0 h post-fertilization (hpf) (single cell stage), 4 hpf (blastula stage), 7 hpf (gastrula stage), 10 hpf (90% epiboly) 13 hpf (blastopore closure and germ ring), 16 hpf, 19 hpf, 22 hpf, 25 hpf (somite/segmentation stage), 31 hpf, 37 hpf (hatching), 43 hpf and 49 hpf (pigmentation of eyes and mouth opening). At this point, 24 h intervals were used to collect samples for RNA extraction and *in situ* hybridization. Each sample consisted of a pool of 100 mg of embryos or larvae. In addition, adult males and females (n = 3 males; 3 females) were euthanized by immersion in water containing benzocaine hydrochloride (250 mg/l). The embryonic and larval development of *R. quelen* is shown in Supplemental material (Supplemental Figs. 1 and 2). Different tissues (brain, gills, heart, liver, muscle, gut, kidney, gonads) were dissected and further processed for RNA extraction or *in situ* hybridization (gonads only).

2.3. Molecular cloning of R. quelen vasa cDNA

Total RNA from testes was extracted according to the FastRNA Pro Green Kit (MP Biomedicals, Solum, OH, USA), following the manufacturer's recommendations. To avoid genomic contamination, RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) prior to cDNA synthesis. The cDNA synthesis was performed with random hexamers using Superscript II (Invitrogen, Carlsbad, CA, USA). Primers *Rqvasa*RT-Fw and *Rqvasa*RT-Rv (Table 1) to specifically PCR amplify a partial *R. quelen vasa* cDNA sequence were based on *vasa* sequences of catfish (*Clarias gariepinus* - NCBI:GU562470) and southern catfish (*Silurus meridionalis* - NCBI: EU532191). To obtain a full-length *R. quelen vasa* cDNA, 5′-RACE and 3′-RACE were performed using the SMART RACE cDNA amplification kit (Clontech, Mountain View,CA, USA), using 2 μg total RNA and gene-specific primers (Table 1) designed based on the partial cDNA sequence obtained above in combination with UPM and NUP primers, supplied with the kit. PCR products were separated by gel electrophoresis and bands of the expected size were gel extracted, cloned into pcDNA3.1/V5-His TOPO vector (Invitrogen, Breda, The Netherlands) and sequenced. A Vasa amino acid sequence was deduced from the full-length *R. quelen vasa* cDNA sequence and aligned with other Vasa sequences, using ClustalW2 tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) at default settings. To determine the possible protein domains, Pfam (http://pfam.sanger.ac.uk/) was used. Phylogenetic analyses were performed by the Neighbor joining (NJ) method, which determine the phylogenetic distance through a heuristic search with 1000 initiation replicates. A consensus cladogram without rooting (MEGA version 6.06) was generated.

2.4. vasa expression: RT-PCR, qPCR and in situ hybridization

vasa expression was determined during embryogenesis, larval development and in several tissues (brain, gills, heart, liver, muscle, gut, eye, gonads) from male and female using reverse transcriptase-PCR (RT-PCR) and real-time, quantitative RT-PCR (qPCR). For all of these methods, total RNA was extracted with Trizol reagent (Life Technologies), followed by DNAse treatment and cDNA synthesis according to standard protocols (Nobrega et al., 2010). RT-PCR and qPCR

Table 1
List of primers used for cloning and expression analysis of *vasa*. RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; ISH, *in situ* hybridization; qPCR, real-time, quantitative polymerase chain reaction.

Primers name	Sequence (5′–3′)	Usage
*vasa*Fw3′-UPM (4156)	CAACCCACCCAAGGCTATCATGACAATCATGACATTTGAAGAA	RACE
*vasa*Fw3′-NUP (4157)	GCCCAATTGTGTGAGACACTGAACAAAAATGTTGCTAAGT	RACE
*vasa*Rv3′-NUP (4167)	ATACTTCTGAACAGGGGTAGGTTTCACGTATCCAGACTTA	RACE
*vasa*Rv3′-UPM (4164)	TTCCCTGATCCAGTCTGGGCACAAGCCATGAGATC	RACE
*vasa*Rv3′-NUP (4165)	CACAAGCCATGAGATCCCTCCCAGCAGATAT	RACE
*vasa*Rv3′-NUP (4166)	CCCAGCAGATATGATGGGAATTCCATACTTCTGAACA	RACE
*vasa*Rv3′-UPM (4163)	ACCTTCATTCATCAGATGCTGCAGAATAGGCAGC	RACE
*vasa*Rv3′-NUP (4159)	CGTTGGTGCCTCCATAAACGACAACAGGACG	RACE
*vasa*Rv3′-UPM (4158)	CACTTCTCGGATTGTAAATCCGACGTTGGTGC	RACE
*Rqvasa*RT-Fw	GGCAGAGGTGGGCGTGGGGGAAG	RT-PCR
*Rqvasa*RT-Rv	CCACGACCAATAATGTCAAGCAATCTTCCAGGG	RT-PCR
RT-*β-actin*-Fw	TGACCTGACTGACTACCTCA	RT-PCR
RT-*β-actin*-Rv	AGCTCATAGCTCTTCTCCAGGGGCGGGTG*TTATTAACCCTCACTAAA*GAGGCT	RT-PCR
*Rqvasa*T3-Fw	ATCATGACATTTGAAGAAG	ISH
*Rqvasa*T7-Rv	CCGGGGGGTG*TAATACGACTCACTATA*GACACAGGTGCCATAAGCA	ISH
Rqvasa-qPCR-Fw	AGGCTATCATGACATTTGAAGAAG	qPCR
Rqvasa-qPCR-Rv	CCATACTTCTGAACAGGGGT	qPCR

were conducted using specific primers for *Rqvasa* and *β-actin* (NCBI: EU527190) (Table 1). For RT-PCR, the amplified products were separated on 1% agarose gel and the expected bands were compared to the molecular weight of the ladder. For qPCR, Cq values for *Rqvasa* were determined using SYBR Green kit (Invitrogen) while Cq values for the reference gene (eukaryotic *18S rRNA*) expression were determined with an Invitrogen assay in combination with a Universal TaqMan kit (Invitrogen). All qPCR reactions (20 µl) were performed in a StepOne system (Life Technologies) following the manufacturer's instructions, and relative gene expression profiles were calculated according to the ΔΔCt method as described previously (Vischer et al., 2003). Riboprobe synthesis for *in situ* hybridization was done using a *R. quelen vasa*-specific PCR product generated with primers *Rqvasa*T3-Fw and *Rqvasa*T7-Rv (Table 1). The 384 bp PCR product was gel purified, and served as a template for digoxigenin (DIG)-labelled cRNA probe synthesis using the RNA labeling (Roche) kit. For *in situ* hybridization, embryo, larvae and gonads were fixed in 4% paraformaldehyde (PFA) in PBS at 4 °C overnight. The protocol used for whole mount (WISH) and *in situ* hybridization (paraffin embedded) were performed with adaptations, as described previously (Thisse and Thisse, 2008). Detection of hybridization signal was done with chromogen BCIP/NBT.

2.5. Light microscopy

For light microscopy, gonads were fixed in modified Karnovsky solution (2% glutaraldehyde and 4% paraformaldehyde in Sorensen buffer [0.1 M, pH 7.2]) for at least 24 h, dehydrated in a graded ethylic series, embedded in Historesin (Leica HistoResin), sectioned (3 µm thickness) and stained with hematoxylin an eosin. The histological sections were examined and documented using Leica DMI6000 microscope (Leica).

2.6. Statistical analysis

Results were expressed as mean values ± SEM. Significant differences between two groups were identified using unpaired Student test ($p < 0.05$). Comparisons of more than two groups were performed with one-way ANOVA followed by Student-Newman-Keuls test ($p < 0.05$). Graph Pad Prism 4.0 (Graph Pad Software, Inc., San Diego, CA, USA, (http://www.graphpad.com) was used for all statistical analysis.

3. Results

3.1. Molecular characterization of Rqvasa cDNA

The full-length *R. quelen vasa* cDNA consists of 2681 bp, comprising a 5′-UTR of 150 bp, open-reading frame of 2016 bp, and a 3′-UTR of 515 bp (Fig. 1). The deduced *R. quelen* Vasa protein is composed of 671 amino acids, containing eight consensus motifs characteristic of the DEAD-box protein family: an N-terminal region rich in glycine (G) and arginine (R) residues – RGG and RG motifs– and a highly acidic C-terminal region composed of tryptophan (W), glutamic acid (E) and aspartic acid (D) (Fig. 1). The DEAD/DEAH box helicase (DEAD) domain is composed of 180 amino acids and the superfamily Helicase C-terminal (Helicase C) of 77 amino acids (Fig. 1). BLAST analysis revealed that DEAD (green box) and Helicase C amino acid sequences showed high similarity with their respective homologs in other animals (Fig. 2). In this work, we did not find any isoform of *R. quelen vasa* mRNA (Supplemental material and methods). Phylogenetic analysis of the DEAD-box protein family revealed that *R. quelen* Vasa segregated with the VASA subfamily and did not cluster with the related proteins P68 or PL10 (Fig. 3). The NJ phylogenetic tree showed that all teleosts were clustered in one clade, suggesting a close relationship in this group with regards to Vasa (Fig. 3). Higher similarity was found between the *R. quelen* Vasa protein and other Vasa proteins in Siluriformes species (*C. batrachus*, *C gariepinus* and *S. meridionalis*) (Fig. 3). In the fish group, Siluriformes formed a sister group with Cypriniformes, and both showed a closer relationship with Salmoniformes and Anguilliformes (Fig. 3).

3.2. Tissue distribution of Rqvasa mRNA

qPCR and RT-PCR analysis of several tissues from adult male and female *R. quelen* showed that *vasa* mRNA was exclusively expressed in the gonads (Fig. 4, Supplemental Fig. 3A). Significantly higher *vasa* mRNA levels were found in ovary than in testis, while other tissues tested did not express detectable *vasa* mRNA (Fig. 4, Supplemental Fig. 3A).

3.3. Expression of R. quelen vasa mRNA during embryonic development and larval stages

The *vasa* expression levels were analyzed during embryogenesis, from zygote to the hatching stage, and during larval phase, from hatching until 264 hpf by qPCR and RT-PCR (Fig. 5, Supplemental Fig. 3B). *vasa* transcripts were detected in the fertilized oocytes (0 hpf),

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                                                        cacagcaggctcacaggaccagct   24
cgacatttactatagccttcttcttagttcaaaccagtctcacagcttcgagagatgtcacag   87
gaccagctcggcatttacagaccattattaaactatctgtatatctctcagctgtctgaacac  150
ATGGAGGACTGGGAAGATGAACAGAGCCCAGTTGTAACCAGCTTAACTCTGGGCAAAGCAGAA  213
 M  E  D  W  E  D  E  Q  S  P  V  V  T  S  L  T  L  G  K  A  E    21
AATGCATGGAACAGCAATGGACAACAAAACGGCAAGGACAATGAAGAAAGTTCTTGGAAACCT  276
 N  A  W  N  S  N  G  Q  Q  N  G  K  D  N  E  E  S  S  W  K  P    42
GGTGCTGGTTTTGGGACTCAGGGGGGAAACAGAGATCGGGGGTTTGGAAAAGTTGATGGAGAC  339
 G  A  G  F  G  T  Q  G  G  N  R  D  R  G  F  G  K  V  D  G  D    63
TTCAAGGGCTTCAGAACAGGAATTGATGAAAATGCAAATGAAGGAGTTGATAATGGCCACTCC  402
 F  K  G  F  R  T  G  I  D  E  N  A  N  E  G  V  D  N  G  H  S    84
TGGAACACTGGTGGAGAGGGCTTTAGCGGACGAGGAGGCAGAGGACGAGGAGGACGAGGAGGA  465
 W  N  T  G  G  E  G  F  S  G  R  G  G  R  G  R  G  G  R  G  G   105
GGAAGAGGATTTAGGAATTCCTTCAAATCTGATGATGAAAATGCGAGTGATGAAGGTTTTAAA  518
 G  R  G  F  R  N  S  F  K  S  D  D  E  N  A  S  D  E  G  F  K   126
AGTGGCTTTAGTGGCAGAGGTGGGCGTGGAGGAAGAGGAGGCCGTGGAGCTTTCCAACAAGGT  581
 S  G  F  S  G  R  G  G  R  G  G  R  G  G  R  G  A  F  Q  Q  G   147
GGTGATGAAGAAGGCAAAGGACGCTTTGGTGGAGGCTATAGAGGACAGAACGAGGAGATATTT  644
 G  D  E  E  G  K  G  R  F  G  G  G  Y  R  G  Q  N  E  E  I  F   168
TCAAAGGGATCACCAAAGGATAACGAGGAAAAAGAAGATGGTGAGACTGCAGGGCCTAAGGTC  707
 S  K  G  S  P  K  D  N  E  E  K  E  D  G  E  T  A  G  P  K  V   189
AACTATATTCCACCGCCACCACCGGAAGAGGAGAACTCCATTTTTTCTCACTACGCAACAGGC  770
 N  Y  I  P  P  P  P  P  E  E  E  N  S  I  F  S  H  Y  A  T  G   210
ATCAACTTTGACAGGTATGATGACATCCTGGTGGATGTAAGCGGAAGCAACCCACCCAAGGCT  833
 I  N  F  D  R  Y  D  D  I  L  V  D  V  S  G  S  N  P  P  K  A   231
ATCATGACATTTGAAGAAGCCCAATTGTGTGAGACACTGAACAAAAATGTTGCTAAGTCTGGA  896
 I  M  T  F  E  E  A  Q  L  C  E  T  L  N  K  N  V  A  K  S  G   252
TACGTGAAACCTACCCCTGTTCAGAAGTATGGAATTCCCATCATATCTGCTGGGAGGGATCTC  959
 Y  V  K  P  T  P  V  Q  K  Y  G  I  P  I  I  S  A  G  R  D  L   273
ATGGCTTGTGCCCAGACTGGATCAGGGAAAACGGCTGCCTTCCTGCTGCCTATTCTGCAGCAT 1022
 M  A  C  A  Q  T  G  S  G  K  T  A  A  F  L  L  P  I  L  Q  H   294
CTGATGAATGAAGGTTTAGCATCCAGCAAGTTCAGTGAGCTGCAGGAGCCTGAAGTCATCATT 1085
 L  M  N  E  G  L  A  S  S  K  F  S  E  L  Q  E  P  E  V  I  I   315
GTTGCGCCCACTCGGGAACTCATTAATCAGATTTACCTAGAAGCCCGCAAGTTTGCTTATGGC 1148
 V  A  P  T  R  E  L  I  N  Q  I  Y  L  E  A  R  K  F  A  Y  G   336
ACCTGTGTGCGTCCTGTTGTCGTTTATGGAGGCACCAACGTCGGATTTACAATCCGAGAAGTG 1211
 T  C  V  R  P  V  V  V  Y  G  G  T  N  V  G  F  T  I  R  E  V   357
TTAAAAGGTTGCAATGTGCTGTGTGGGACCCCTGGAAGATTGCTCGACATTATTAACCGTGGA 1274
 L  K  G  C  N  V  L  C  G  T  P  G  R  L  L  D  I  I  N  R  G   378
AAGGTTGGATTAAGTAAAATTCGTTTTTTGGTGCTGGATGAAGCTGATCGAATGTTGGATATG 1337
 K  V  G  L  S  K  I  R  F  L  V  L  D  E  A  D  R  M  L  D  M   399
GGATTTGAGGCGGACATGCGAAAGCTGGTAAACTCTCCAGGAATGCCTTCTAAAGAAGAGCGA 1400
 G  F  E  A  D  M  R  K  L  V  N  S  P  G  M  P  S  K  E  E  R   420
CAAACCCTTATGTTCAGTGCCACTTACCCGGAAGATATTCAGAAGCTGGCAGCCGACTTCCTA 1463
 Q  T  L  M  F  S  A  T  Y  P  E  D  I  Q  K  L  A  A  D  F  L   441
AAGGTGGATTATCTGTTCCTGGCTGTGGGAGTAGTGGGAGGAGCCTGTAATGACATTGAGCAG 1526
 K  V  D  Y  L  F  L  A  V  G  V  V  G  G  A  C  N  D  I  E  Q   462
CACATCATTCAGGTCACTCAGTACTCAAAGAGGGAAAAGCTACTGGATCTGCTAAAGACTACA 1589
 H  I  I  Q  V  T  Q  Y  S  K  R  E  K  L  L  D  L  L  K  T  T   483
GGGACACAGAGAACAATGGTCTTTGTTGAAACGAAACGAAGTGCAGATTTCATTGCAACATTC 1652
 G  T  Q  R  T  M  V  F  V  E  T  K  R  S  A  D  F  I  A  T  F   504
CTCTGTCAGGAAAAAATGCCCACTACAAGCATCCATGGCGATCGTGAACAGCGAGAGCGAGAG 1715
 L  C  Q  E  K  M  P  T  T  S  I  H  G  D  R  E  Q  R  E  R  E   525
ACGGCTCTCCGTGATTTCCGCACAGGCCAATGTCCTGTGCTGGTAGCTACTTCTGTTGCTGCT 1778
 T  A  L  R  D  F  R  T  G  Q  C  P  V  L  V  A  T  S  V  A  A   546
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Fig. 1. Nucleotide and deduced amino acid sequences of *R. quelen vasa*. Nucleotides and amino acids are numbered on right. Conserved sequences within the Vasa protein are indicated in boxes. The arginine-glycine (RG) and arginine-glycine-glycine (RGG) repeats are indicated in the N-terminal region (bold and underlined). The C-terminal region composed by acidic amino acids, such as tryptophan (W), glutamic (E) and aspartic acid (D) (bold) are also indicated. The start and stop codons (ATG and TAG) are indicated with white characters on a black background. The polyadenylation signal (aataaa) and the poly-A tail are underlined. The sequence was deposited at GenBank (NCBI) with the access number KF640082.

which corresponds to the zygote stage (Fig. 5, Supplemental Fig. 3B). Following embryogenesis, from the first embryo cleavage (4 hpf) to gastrulation, a significant decrease in *vasa* expression levels was observed (Fig. 5, Supplemental Fig. 3B). During gastrulation (13 hpf), somitogenesis (16–37 hpf) and larval stages (43–264 hpf), the relative expression of *R. quelen vasa* remained constant (Fig. 5, Supplemental Fig. 3B). The Cq values of *vasa* transcripts were normalized with *18S rRNA* levels and expressed as values relative of the values at 0 hpf (Fig. 5, Supplemental Fig. 3B). For comparative analysis, relative *vasa* mRNA levels in the adult testis are also shown (Fig. 5, Supplemental Fig. 3B).

3.4. Localization of *R. quelen vasa* mRNA during embryonic and larval stages by WISH

R. quelen vasa mRNA sites of expression were identified during embryonic and larval stages through chromogenic WISH. WISH showed that *vasa* transcripts were expressed in a restricted subset of cells distributed randomly around the blastoderm margin of a 90% epiboly stage embryo (gastrula - 10 hpf) (Fig. 6A,B). The *vasa*-expressing cells migrate through the shield and translocate from the epiblast to the hypoblast (Fig. 6C). During somitogenesis (19 hpf), *vasa* mRNA was found in the PGCs which were associated to the yolk syncytial layer at

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AGAGGACTAGACATTGAGCATGTCCAGCATGTGGTGAACTTTGACCTGCCTAAAGAAATTGAT 1841
 R  G  L  D  I  E  H  V  Q  H  V  V  N  F  D  L  P  K  E  I  D   567
GAGTATGTGCACCGCATTGGGAGAACGGGCCGATGTGGAAACACAGGAAGAGCCGTGTCCTTT 1904
 E  Y  V  H  R  I  G  R  T  G  R  C  G  N  T  G  R  A  V  S  F   588
TTTGACCCGGAGGCCGATACTCCGTTGGCCCGCTCTCTGGTCAAAGTCCTTTCCGAGGCCCAG 1967
 F  D  P  E  A  D  T  P  L  A  R  S  L  V  K  V  L  S  E  A  Q   609
CAGGAAGTTCCTTCATGGCTGGAGGAAATTGCATTGGGTGCTCACGGTACCACAGGGTTTAAC 2030
 Q  E  V  P  S  W  L  E  E  I  A  L  G  A  H  G  T  T  G  F  N   630
CCACGTGGTAAAGTGTTTGCCTCAACTGACTCTCGCAGGGGTGGATCCTTTACAAAGAACCTG 2093
 P  R  G  K  V  F  A  S  T  D  S  R  R  G  G  S  F  T  K  N  L   651
GCACCACAACCAGCTGCACAAAGCACCGTCACTGCTGCCGCTGATGACGACGACTGGGAGTAG 2156
 A  P  Q  P  A  A  Q  S  T  V  T  A  A  A  D  D  D  D  W  E       672
attgactttttttttgttttttgttgtgcggtattcctctgttcatttttttatacatattt 2219
tatgttaaataagaggcctatgggcccaaaagtataaagagaagagaatggcaggtcctgagc 2282
tatggcactgaaagagccacaggttcaacctgaagttaagcttcacaatttaatgttagtaat 2345
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aaaaaaaaaaa                                                     2671
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Fig. 1. (*continued*)

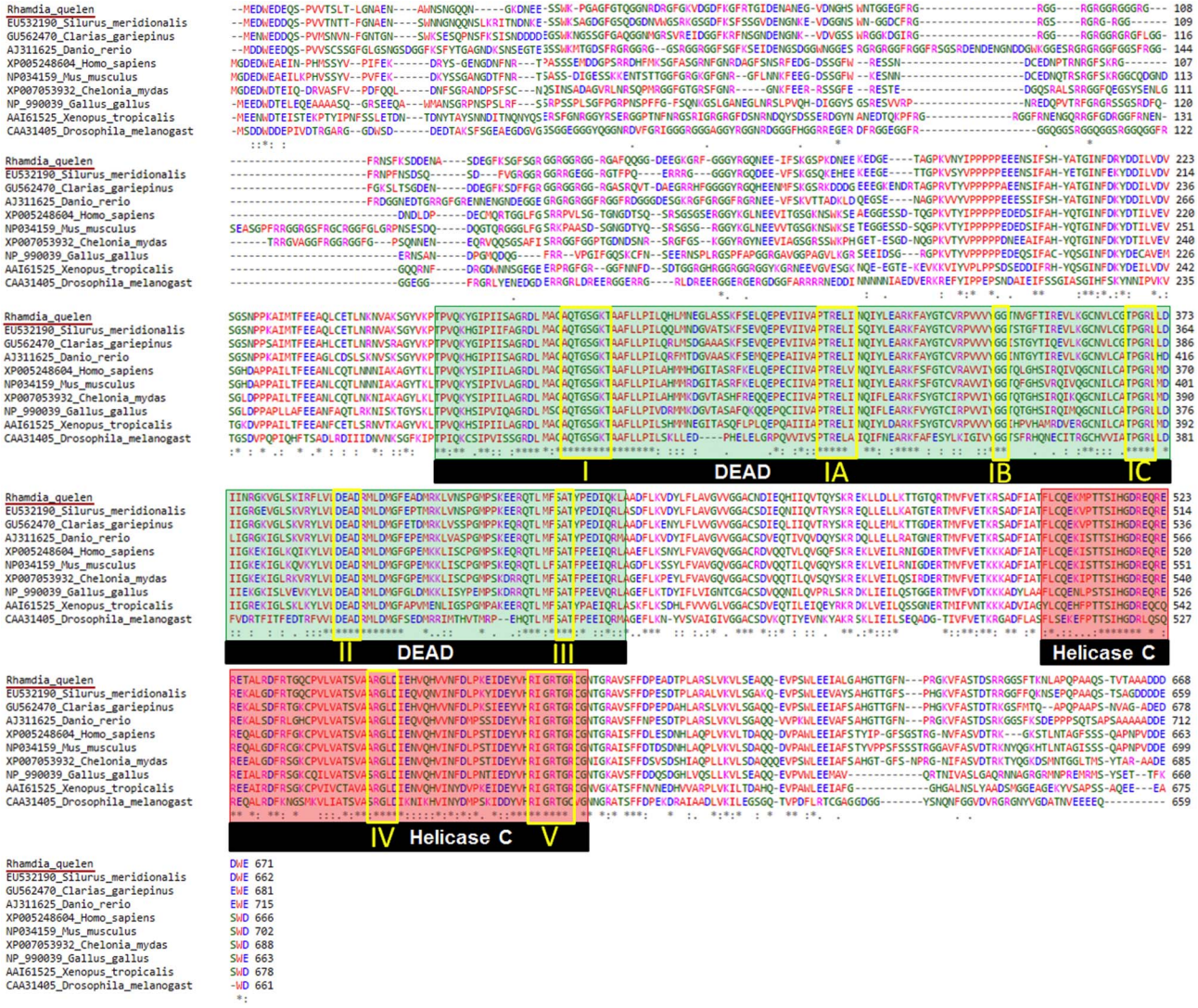

Fig. 2. Comparison between the *R. quelen* Vasa amino acid sequence with Vasa homologs from different organisms. The alignment was performed using the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Asterisks (*) showed conserved sites of the amino acid; two points (:) a strong score, and one point (.) a weak score according to comparison ClustalW alignment. The DEAD-box sequence is indicated in green, while the Helicase C is in red. The eight domains within these superfamilies [Domain I (AQTGSGKT)], IA domain (PTREL), IB domain (GG), IC domain (TPGRL), Domain II (DEAD), domain III (SAT), domain IV (ARGLD) and the V domain (GRTGR) are shown by yellow rectangles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

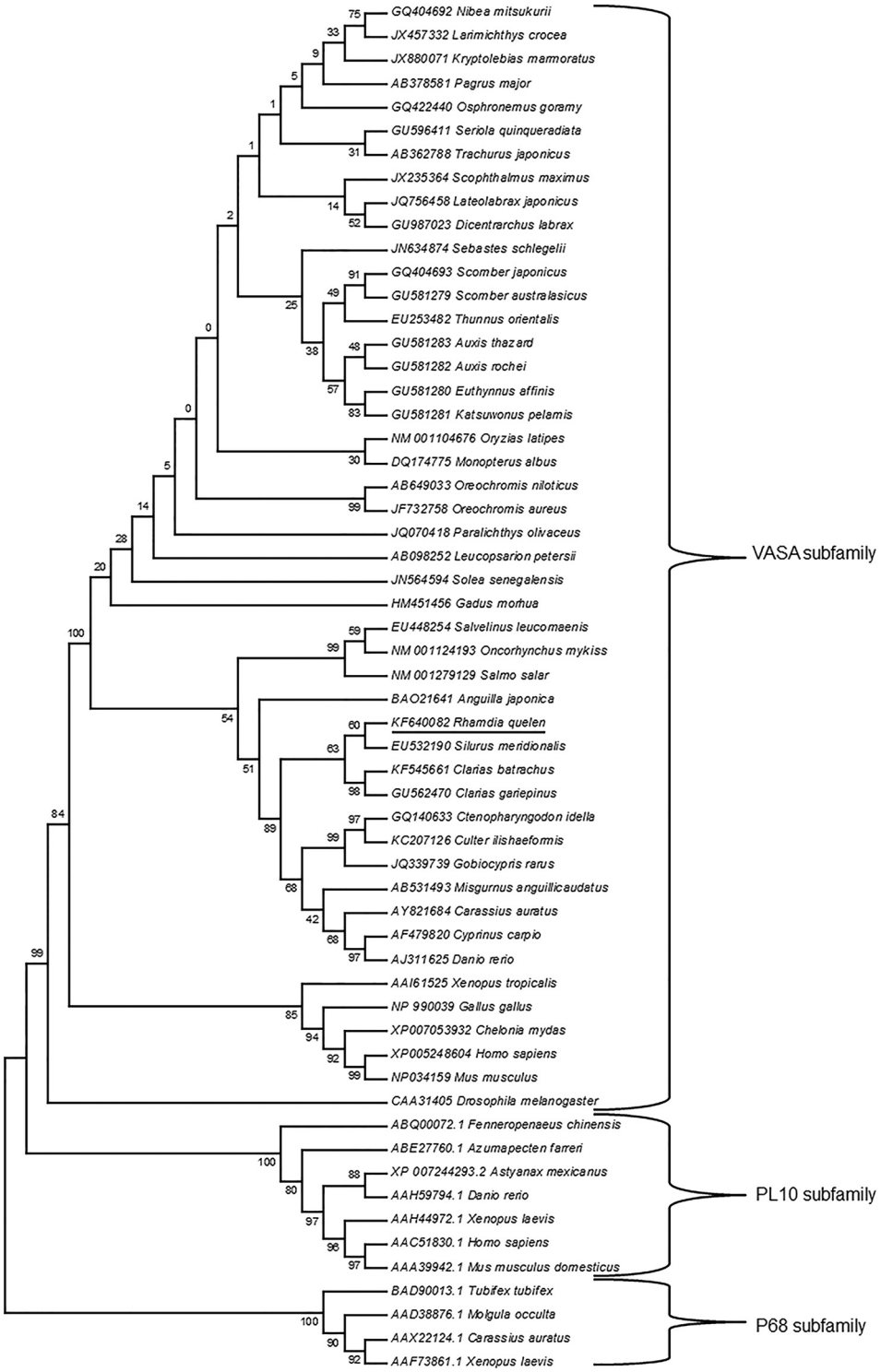

Fig. 3. Phylogenetic analysis of Vasa family protein members. The branch lengths are drawn to scale to the evolutionary distance based on Neighbor-Joining method, and the numbers are the percentage of bootstrap values supporting each node from 1000 replicas (the number listed above of the branch showed the statistic sustentation of the relation, 50 > or = indicating a well support). The GenBank accession numbers were shown to the each species. The underlined *R. quelen* Vasa was clustered in the VASA subfamily, and not with other DEAD-box protein family members, such as PL10 and P68 subfamilies.

this stage (Fig. 6C). In the following stages (37, 43, 55, 72, 96 and 120 hpf), *vasa* transcripts were found in PGCs during their migratory process to the future gonad of the larva through the dorsal mesentery (Fig. 6D–L).

3.5. Cellular localization of *R. quelen vasa* mRNA expression in the gonads

Identification of specific cell types expressing the *R. quelen vasa* mRNA was accomplished by chromogenic *in situ* hybridization using ovary and testis paraffin-embedded sections of ovary and testis tissue (Figs. 7 and 8). Some ovaries were also subjected to WISH for detection of *vasa* transcripts (Fig. 7G). In the ovary, *R. quelen vasa* was mainly

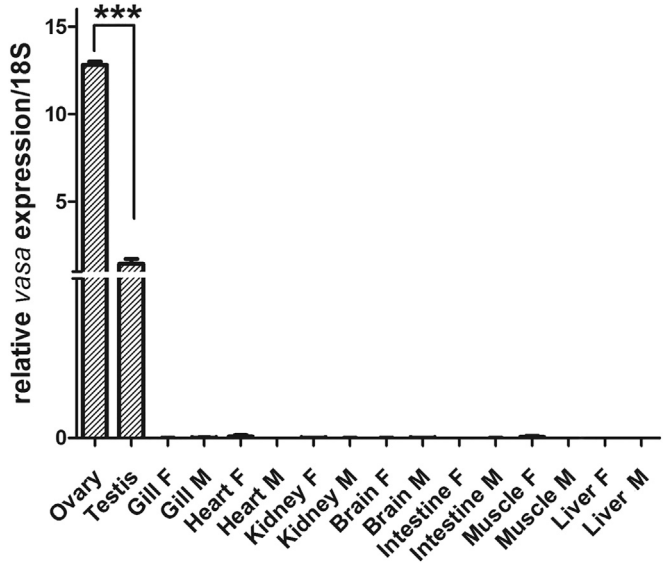

Fig. 4. Relative expression of *R. quelen vasa* mRNA in different adult tissues/organs from male (M) and female (F). cDNA from various tissues of adult fish (gill, heart, kidney, brain, intestine, muscle, testis, and ovary) were used for qPCR. The expression levels were normalized to the expression of *18S rRNA* (reference gene). Values represent mean ± SEM relative to testicular *vasa* mRNA levels. The asterisks indicate a significant difference between testicular and ovarian tissue ($p < 0.05$).

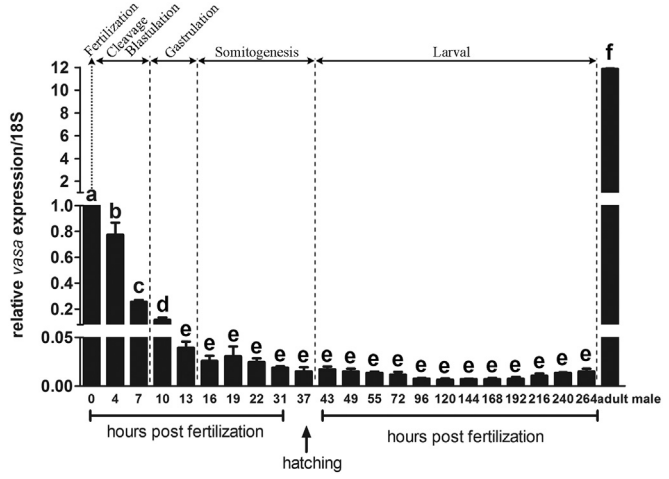

Fig. 5. The relative expression of *R. quelen vasa* mRNA during embryonic and larval development of *R. quelen* by qPCR. RNA was extracted from whole embryos at different stages of development from unfertilized eggs to 264 h post-fertilization (hpf). The expression of *vasa* mRNA was normalized to *18S rRNA* and expressed as relative values to *vasa* mRNA levels at 0 hpf. Expression of *R. quelen vasa* in testis from adult males is also shown. Data are expressed as mean ± SEM. Different letters denote significant difference between each other ($p < 0.05$, one-way ANOVA).

expressed in the perinucleolar oocyte stage (Fig. 7 D, E, F, G). In the early perinucleolar oocyte, *vasa* transcripts were found in the cytoplasm, and also in the nucleus, which showed a strong staining (Fig. 7D, F, G). In the late perinucleolar oocyte, *vasa* mRNA was found in the nucleolus, cytoplasm and in the Balbiani body (Fig. 7E). In the previtellogenic oocytes, *R. quelen vasa* was distributed uniformly in the cytoplasm (Fig. 7F). The signal is evidently less intense in vitellogenic oocytes, because of the decrease of *vasa* mRNA expression or its dispersion (Fig. 7E, F). In the WISH, the early germ cells are the ones presenting high intensity of *vasa* expression (Fig. 7G).

In the testis, *vasa* transcripts were exclusively expressed in the germ cells at different stages of spermatogenesis (Fig. 8 D, E, F). Germ cells develop within spermatocysts (or cysts), which are distributed along the germinal epithelium (Fig. 8 D,E). Spermatogonia (from type A until B) presented the strongest signal for *vasa* mRNA, showing a mild decrease in intensity from type A spermatogonia to spermatids, and no signal in spermatozoa (Fig. 8 F).

4. Discussion

In the current study, we have isolated and characterized the full-length *vasa* cDNA of a neotropical catfish species, *Rhamdia quelen*, and analyzed its expression profiles during embryogenesis and larval development. The deduced *R. quelen* Vasa protein has a Helicase C superfamily domain in the C-terminal and a DEAD-box in the N-terminal, as observed in *D. labrax* (Blazquez et al., 2011) and *P. olivaceus* (Wu et al., 2014). Both domains are typical of the Vasa protein family. The superfamily DEAD and Helicase C sequences of *R. quelen* Vasa shared high similarity with their respective homologs in other animals. Vasa, PL10 and P68 subfamilies are important DEAD-box protein family members. Phylogenetic analysis of Vasa proteins involved other DEAD-box proteins from both vertebrates and invertebrates. Our results revealed that the *R. quelen* Vasa most closely resemble the Vasa subfamily instead of the other DEAD-box protein family members, such as the P68 and PL10 subfamilies, as shown in *Cynoglossus semilaevis* by Wang et al. (2014). Phylogenetic analysis showed that *R. quelen* Vasa is clustered with Vasa of other Siluriformes, such as *C. batrachus*, *C. gariepinus* and *S. meridionalis*. Moreover, our phylogenetic tree demonstrated that Siluriformes, Cypriniformes, Salmoniformes and Anguiliformes are evolutionarily related, indicating a monophyletic condition for the Vasa protein. In this work, we have not found other isoform for *vasa* mRNA in *R. quelen* gonads (see Supplemental material and methods), similarly as reported in the catfish *C. gariepinus* (Siluriformes) (Raghuveer and Senthilkumaran, 2010). *S. meridionalis* is the only catfish reported until now with different *vasa* isoforms in the gonads (Hu et al., 2008). Therefore, this work is the first one to characterize *vasa* mRNA from a neotropical catfish, showing one form of *vasa* transcript. More studies will be necessary to address if other neotropical catfish species do have one form of vasa mRNA or not.

We also evaluated the tissue distribution of *vasa* in male and female by RT-PCR and qPCR. *vasa* mRNA was predominantly expressed in the gonads, significantly higher in ovaries than in the testes. Similar results were found in invertebrates, such as *Caenorhabditis elegans* (Gruidl et al., 1996), *D. melanogaster* (Hay et al., 1988), and in vertebrates, as *D. rerio* (Yoon et al., 1997), *C. gariepinus* (Raghuveer and Senthilkumaran, 2010), *P. olivaceus* (Wu et al., 2014), *Xenopus* (Ikenishi and Tanaka, 2000) and others. In the gonads, *vasa* transcripts were found to be exclusively expressed in germ cells, as reported previously in *D. melanogaster* (Lasko and Ashburner, 1988), zebrafish (Yoon et al., 1997), rainbow trout (Yoshizaki et al., 2000), tilapia (Kobayashi et al., 2000), *Xenopus* (Komiya et al., 1994; Ikenishi and Tanaka, 2000), chicken (Tsunekawa et al., 2000) and others.

Interestingly, *vasa* was strongly expressed in germ cells at early stages of development in *R. quelen* gonads, as observed in other teleosts (Braat et al., 1999; Knaut et al., 2000; Xu et al., 2005), and decreased its expression as gametogenesis progresses. In spermatogenesis, *R. quelen vasa* showed a mild decrease in expression from type A spermatogonia to spermatids, and no signal in spermatozoa. Similar results were found in *A. japonicus* (Xu et al., 2005) and *C. auratus gibelio* (Xu et al., 2005), although in the latter one, *vasa* was not present in spermatids either (Xu et al., 2005). On the other hand, in *Oreochromis niloticus*, *vasa* mRNA was detected in all stages of germ cell development, from type A spermatogonia to spermatozoa (Kobayashi et al., 2000). Such variation of *vasa* expression in different species might be related to the function of *vasa* in spermatogenesis, however, very few information was obtained in the last years in this regard. In medaka, analysis of the Vasa protein during spermatogenesis suggests that this protein has an important role in cytodifferentiation and formation of the sperm tail during spermiogenesis (Yuan et al., 2014). This hypothesis was based due the presence of Vasa in the chromatoid body, which is a ring-

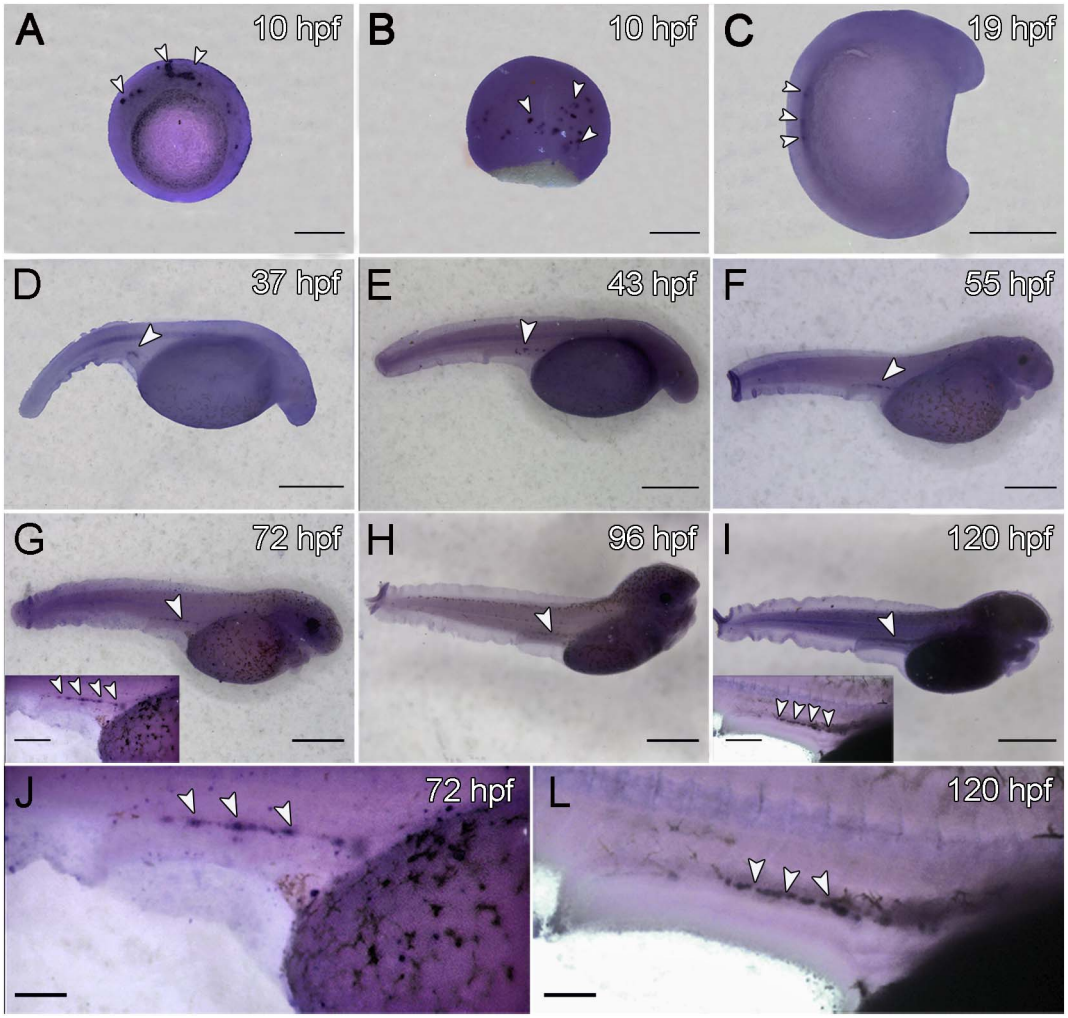

Fig. 6. Whole mount *in situ* hybridization (WISH) of *R. quelen vasa* mRNA during the embryonic and larval development of *R. quelen*. Ventral (A) and lateral (B) view of an embryo at 10 h post-fertilization (hpf) (gastrulation stage - 90% epiboly). The dark spots (arrowheads) indicate *R. quelen vasa* expressing cells. (C) Embryo at 19 hpf stage showing *vasa* expressing PGCs associated to the yolk syncytial layer. (D, E, F, G, H, I, J, L) During the larval development, *R. quelen vasa* is expressed in PGCs (arrowheads), which are located at the dorsal mesentery migrating towards the developing gonadal ridge. Bars: A–I = 500 μm; J, L = 100 μm.

shaped structure surrounding the tail of the spermatids (Yuan et al., 2014). Interestingly, although the role of the chromatoid body is unclear, studies in mouse have demonstrated that the loss of the ring-shaped structures resulted in male infertility (Shang et al., 2010). Moreover, recent studies showed that *vasa* is essential for spermatogenesis and not for oogenesis; *e.g.* loss of *vasa* homolog in mice and zebrafish resulted in defective spermatogenesis and male sterility, while oogenesis remained normal (Tanaka et al., 2000; Shang et al., 2010; Hartung et al., 2014).

In ovaries, *R. quelen vasa* is highly expressed in the perinucleolar oocyte, specifically in the cytoplasm, nucleus, nucleolus and in the Balbiani body. At the previtellogenic stage, *R. quelen vasa* is expressed in the cytoplasm, decreasing its expression in the vitellogenic oocyte. Such decrease is similar to the one found in *L. calcarifer* (Xu et al., 2014), *O. niloticus* (Kobayashi et al., 2000), *A. japonicus* (Yan et al., 2013) and *C. auratus gibelio*, (Xu et al., 2005). Interestingly, in human (*Homo sapiens*), VASA protein was found in the primary follicles at the pubertal stage, and as follicle starts its growing, the protein is no longer detected (Albamonte et al., 2013). This variation of *vasa* expression during female germ cell development could be associated to processes of self-renewal, differentiation and meiosis (Xu et al., 2014). However, more studies are needed to unravel the role of *vasa* in oogenesis. Interestingly, in some organisms, this gene is essential for oogenesis. In *D. melanogaster*, for example, mutations in maternal inherited genes, such as *vasa*, showed that females did not complete oogenesis and are sterile (Schupbach and Wieschaus, 1991). On the other hand, in teleosts, *vasa* seems to be dispensable for female germ cell development, as mentioned above.

Changes in *R. quelen vasa* mRNA expression were analyzed from 0 to 264 hpf in whole *R. quelen* embryos to investigate whether *vasa* mRNA is among the maternally contributed mRNAs and when *R. quelen vasa* mRNA expression starts during early embryonic development. In *R. quelen* embryos, *vasa* mRNA could be detected at 0 hpf, which suggests that *vasa* is maternally deposited in oocytes. Usually, maternally inherited factors (proteins, mRNAs) are deposited during oogenesis in structures so-called as germinative plasm and Balbiani body (Lasko and Ashburner, 1988). In this work, we localized the *R. quelen vasa* primary transcripts in Balbiani bodies at the perinucleolar stage (see above). The Balbiani body is a typical structure of female germinative cells, generally composed of mitochondria and electron dense granules with long duration RNAs (such as *vasa* mRNA) and proteins (Voronina et al., 2011). These structures remain along the embryo development, being responsible for the specification of the germ cell lineage in different organisms, such as *D. melanogaster* (Lasko and Ashburner, 1988), medaka (*O. latipes*) (Herpin et al., 2007) and others. In line with this, many studies have demonstrated maternally inherited *vasa* transcripts in oocytes (cytoplasm and Balbiani body) and early embryonic development stages in teleosts. In zebrafish, for example, *vasa* mRNA is detected very

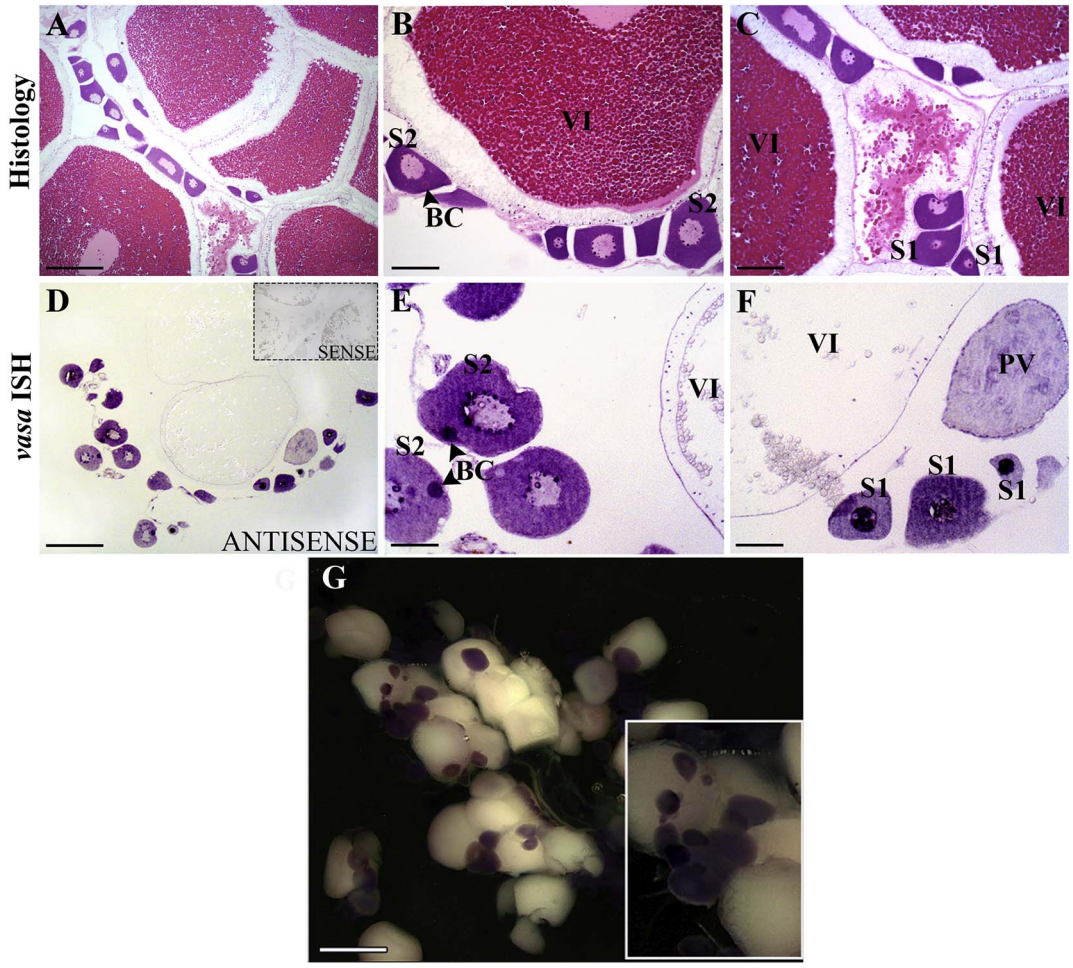

Fig. 7. Histological sections of ovarian tissue of *R. quelen*. (A–C). Cross section stained with hematoxylin and eosin (HE) showing the different stages of oocyte development in *R. quelen*. (B) Arrowhead indicates the Balbiani corpuscle (BC) in the late perinucleolar oocyte stage (S2). Identification of *R. quelen vasa* mRNA expression sites in *R. quelen* ovaries through chromogenic *in situ* hybridization using paraffin embedded sections with either antisense probe (T7) (D, E, F) or sense probe (T3) (D-inset). (D-inset) The sense probe showed the specificity of the reaction for the riboprobes used. (E) *vasa* mRNA was found in perinucleolar oocytes, specifically in the cytoplasm, Balbiani corpuscle and in the nucleus and nucleoli. (F) Intense staining in the nucleus showed high expression of *vasa* primary transcripts in the early perinucleolar oocyte stage (S1). In the previtellogenic oocyte (PV), *R. quelen vasa* was distributed uniformly in the cytoplasm. (G) Whole mount *in situ* hybridization of *R. quelen vasa* mRNA in *R. quelen* ovary. S1 = early perinucleolar oocyte stage, S2 = late perinucleolar oocyte stage, PV = previtellogenic, VI = vitellogenic. Bars: A, D = 250 μm; B, C = 100 μm; E, F = 50 μm; G = 1000 μm.

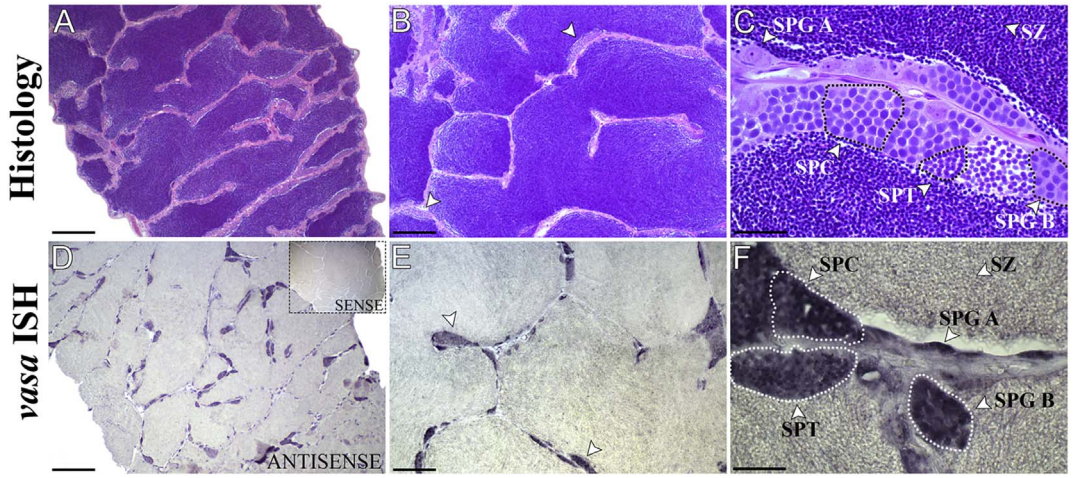

Fig. 8. Histological analyses and chromogenic *in situ* hybridization for the cellular localization of *vasa* mRNA in *R. quelen* testis. (A–C) Histological section of sexually mature testis stained with hematoxylin and eosin (HE). Note that the lumen contains abundant spermatozoa. (C) Detail of a germinal epithelium showing cysts with different stages of germ cell development; spermatogonia type A (SPG A), spermatogonia type B (SPG B), spermatocytes (SPC), spermatides (SPT) and spermatozoa (SZ). (D,E, F) Hybridization using antisense probe (T7). Arrowheads indicated *R. quelen vasa* expressing cells in the *R. quelen* testis. (D-inset) Hybridization using sense probe (T3) as negative control. (E) Arrowheads showed that *vasa* is expressed in germ cells at different developmental stages of spermatogenesis. (F) Higher magnification of the germinal epithelium; *R. quelen vasa* is strongly expressed in germ cells at early stages of development. Bars: A, D = 250 μm; B, E = 100 μm; C, F = 25 μm.

early during embryonic development, and embryonic *vasa* mRNA expression starts only at the medial blastula (Kane and Kimmel, 1993;Yoon et al., 1997). In this work, the presence of *R. quelen vasa* mRNA in oocyte (cytoplasm and Balbiani body) is another evidence of maternal inheritance of this transcript, which will contribute for the germline establishment in the embryos of this species.

The relative *R. quelen vasa* mRNA levels decreased significantly and gradually from 0 to 13 hpf, where it reached its lowest levels of expression and remained constant along the evaluated period (264 hpf). Interestingly, the lowest *R. quelen vasa* expression was also observed in the same period of the embryonic development of Japanese flounder (Wu et al., 2014), *D. rerio* (Wolke et al., 2002), gibel carp (Xu et al., 2005) and *C. gariepinus* (Raghuveer and Senthilkumaran, 2010). This observation might be explained due to maternal transcripts degradation or by the dilution of *vasa* transcripts because of the increased number of cells in the organism. The cellular sites expressing *R. quelen vasa* were identified in the whole embryo by WISH. We have observed that *R. quelen vasa* primary transcripts were expressed exclusively in the migrating PGCs. Thus, at the 10 hpf (90% epiboly), PGCs were present at the dorsal region of the embryo, further translocating from the epiblast towards the hypoblast, similar as in *Gymnogobius* species (Saito et al., 2002). In the somitogenesis stage, PGCs were located at the mesoderm, in association with the syncytial layer of the yolk, as reported previously (Nagai et al., 2001; Saito et al., 2002; Saito et al., 2004). At the advanced larval stages (37, 43, 55, 72, 96 and 120 hpf), the *vasa* mRNA expressing PGCs were located in the dorsal mesentery, most likely in a migratory process towards the genital ridge. Such observation is similar to studies in zebrafish, where PGCs migration was identified at the five somite phase, at the border of the endoderm and contacting the syncytial layer of the yolk sac, followed by its migration from the dorsal mesentery to the genital ridge (Braat et al., 1999).

In summary, the current work has characterized the full-length *vasa* cDNA from a neotropical catfish species, *R. quelen*. We showed high similarity between the predicted amino acid sequence encoded by *vasa* and the other Vasa homologs, especially in the typical DEAD-box and Helicase C superfamily domains. *R. quelen vasa* mRNA expression seemed to be restricted to gonads, specific of the germ cell lineage. Expression analysis during the embryonic and larval development showed that *vasa* is maternally inherited and later is expressed in PGCs during their migratory process to the gonadal ridge. Our results contributed for basic knowledge of *R. quelen* reproductive biology, showing that *R. quelen vasa* is an useful germ cell marker for biotechnological studies, such as germ cell transplantation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2018.02.029.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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